Hide and seek: a multidisciplinary study on the ecological success of an estuarine dweller

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Image of a black and a white brown shrimp *Crangon crangon* (L.) after acclimation on black and white sediment, respectively. Pigments are visible within chromatosomes beneath the translucent exoskeleton. The location of the stomach is also visible below the carapace of the white shrimp.

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To my mother, Annelies Goossens, and my grandmother, Dorothea Blok, who sparked my passion and love for nature

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List of abbreviations

12S: Mitochondrial 12S rRNA gene
AIC: Akaike Information Criterion
ALT: Alternating backgrounds
ANOVA: Analysis Of Variance
BL: Black beaker
CCA: Canonical-Correspondence Analyses
CI: Chromatophore Index
CL: Carapace Length
COI: mitochondrial Cytochrome *c* Oxidase subunit I
CST: Constant background
CTR: Control Treatment
dw: Dry weight
envDNA: Environmental DNA
GLM: Generalised Linear Models
HTS: High-Throughput-Sequencing
ICP-OES: Inductively Coupled Plasma Optical Emission Spectrometry
ITS: Internal Transcribed Spacer
MI: Melanophore Index
MOTU: Molecular Taxonomic Unit
PCA: Principal Component Analysis
PCR: Polymerase Chain Reaction:
PERMANOVA: Permutational Multivariate Analysis Of Variance
PiC: Pigment Cover
TL: Total Length
TLC: Time since change in artificial light status
TOM: Total Organic Matter
UD: Under limit of Detection
WH: White beaker

A glossary of key terms is included in Appendix 1.

Abstract

Estuaries are dynamic systems in which biotic and abiotic conditions vary remarkably. Survival in these habitats requires flexibility in dealing with these variations, which include changes in food availability and shelter opportunities. A thorough understanding of ecological networks in these systems, therefore, relies on comprehensive information on anti-predator adaptations and trophic relationships among species. The present study focuses on two key traits of the brown shrimp *Crangon crangon* L. (Decapoda: Caridea), a key component of European sandy shores, namely i) its ability to conceal from predators and ii) its trophic flexibility. This shrimp is well known for its camouflage abilities and central role in the estuarine food web. Here, I applied behavioural experiments using a novel method to quantify pigment cover (PiC) to study background matching in *Crangon crangon*, and assessed the application of metabarcoding to define its trophic ecology in six European estuaries. Results indicate that the brown shrimp is capable of repeated fast colour adaptations and that its background matching ability is mainly influenced by presence of light and sediment colour. High levels of intra- and inter-individual variation indicated, on the other hand, a complex balance between behavioural-plasticity and environmental adaptation. Large spatial variation, on local and regional scales, in its diverse diet also confirmed the highly flexible nature of this trophic opportunist. Its diet reflected local patterns in prey item distributions, and the variation in its stomach content was evaluated as a tool for the assessment of heavy metal pollution impacts and fish biodiversity patterns in European estuaries for the first time. Overall, the results of this study yielded insights into many key factors influencing predator-prey dynamics in estuarine systems. This included the effects of environmental and behavioural factors on the evolution of animal background matching and the application of metabarcoding towards a more robust reconstruction of ecological networks.

Chapter 1.

Introduction and background

1.1. Introduction

Being located at the interface between the terrestrial and marine world, estuarine systems are a unique dynamic habitat characterised by high variability and large gradient in biotic and abiotic factors (Ysebaert et al., 2003, Elliott and Quintino, 2007). These habitats are of great ecological and economical importance. For example, they play an important role in carbon and nutrient recycling, act as nursery grounds and refuge for many ecologically and economically important species and are essential for coastal protection (Hyndes et al., 2014, Martínez et al., 2007, Sheaves et al., 2015). They are under large anthropogenic pressure, nevertheless, including eutrophication, overfishing, habitat destruction and can act as a sink for heavy metals and other pollutants (Teuchies et al., 2013, Kennish, 2002). These high levels of natural and anthropogenic variation results in a system in which only a subset of species are adapted to, compared to the adjacent marine and fresh water habitats (Elliott and Quintino, 2007, Chapman and Wang, 2001).

Within estuaries, soft-bottom habitats are of special interest since the low structural complexity of these habitats provides limited refuge opportunities from predation and reduced chances to decrease competition for epibenthic animals (animals living on the surface of the bottom of a water body; Gilinsky, 1984, Diehl, 1992, Moksnes et al., 1998). Epibenthic animals find refuge from predation in these habitats in shallow areas, in schools of conspecifics, or by means of burying or crypsis (Ruiz et al., 1993, Pinn and Ansell, 1993, Ryer et al., 2008, Ferrell and Bell, 1991). These soft-bottom habitats are, however, often located in high energy areas such as surf zones or are influenced by anthropogenic disturbances such as bottom-trawling, which can result in the rapid exposure of sheltered prey items (Lasiak, 1984, Hewitt et al., 1997, Queirós et al., 2006). High flexibility in the anti-predator behaviour of these intertidal animals is therefore required to survive in these dynamic unvegetated habitats (Nanjo et al., 2011, Laurel and Brown, 2006).

The dynamic nature of these habitats also influences the behaviour of predators, with the majority of them showing opportunistic feeding behaviour in response to the high biotic and

abiotic variability of these systems (Pihl, 1985, Beyst et al., 2001, Lasiak, 1984, Ansell et al., 1999, Lasiak and McLachlan, 1987). Survival in these complex habitats thus requires flexibility in dealing with environmental variability, both in relation to changes in shelter and food availability. A thorough understanding of these predator-prey relationships is essential for the study of ecological networks, population dynamics and anthropogenic impacts in estuaries (Evans et al., 2016a, Joachim et al., 2017, Van Tomme et al., 2014). This is especially the case for soft-bottom communities since the structuring role of epibenthic predators in these communities is complex and still under discussion (Ambrose, 1984, Thrush, 1999, Gee et al., 1985, Evans, 1983, Van Tomme et al., 2014). The following sections will discuss (a) the study organism of this thesis, the brown shrimp, *Crangon crangon* (L.), (b) the importance of camouflage and colour change as anti-predator strategies, and (c) the application of metabarcoding to study predator-prey relationships.

1.2. The study organism: *Crangon crangon*

The brown shrimp, *Crangon crangon* (L.) (*Decapoda: Caridea*; Figure 1.1), is a widespread and abundant crustacean in European coastal and estuarine habitats (Campos and van der Veer, 2008, Henderson et al., 2006). It has been used as a model species in a wide range of studies encompassing topics such as ecophysiology, toxicology, predator-prey interactions and behavioural studies (Hunter et al., 1998, Pinn and Ansell, 1993, Koller, 1927, Wennhage, 2002, Elofsson and Kauri, 1971). The brown shrimps' well-studied ecology and physiology, ecological and economical importance and ease of catch are some of the factors playing a role of in its suitability for biological research (Campos and van der Veer, 2008, Tiews, 1970).

Figure 1.1. Photo of *Crangon crangon*. Pigments are visible within chromatosomes beneath the translucent exoskeleton. The location of the stomach is also visible below the carapace.

1.2.1. Geographical distribution and habitat

The brown shrimp is a widely distributed and highly abundant decapod crustacean in the North-East Atlantic, Western Mediterranean, Adriatic Sea and Black Sea (Campos et al., 2009a, Campos et al., 2009b, Tiews, 1970, Cattrijsse and Makwaia, 1994). Its population is divided into four major phylogenetic groups (north-eastern Atlantic, western Mediterranean and the Adriatic and Black Seas) with restricted gene flow between these groups (Luttikhuizen et al., 2008). It occurs in soft bottom substrates ranging from sand to mud, with a preferred grain size between 125-710 µm (Pinn and Ansell, 1993, Lloyd and Yonge, 1947). *Crangon crangon* is the dominant species in the surf zone of sandy beaches, being present throughout the year (Beyst et al., 2001, Gibson et al., 1993). Habitat segregation with other common crustaceans has been noted in some cases (Bamber and Henderson, 1994), but *C. crangon* co-occurs often with *Carcinus maenas*, another major component of the European sandy shores (Beyst et al., 2001, Gibson et al., 1993).

1.2.2. Life history

The life cycle of *C. crangon* includes seasonal, size- and sex-specific migrations. Reproduction takes place offshore (up to 20 m depth; Bamber and Henderson, 1994, Henderson and Holmes, 1987, Allen, 1960). Egg development is temperature dependent and takes 2.5 to 13 weeks (Kuipers and Dapper, 1984). Larvae hatch at around 2 mm length, but both the egg size and larval development are season dependent to allow for extra starvation resistance during the winter months (Paschke et al., 2004, Lloyd and Yonge, 1947). The larvae go through five pelagic stages while migrating inshore to settle in tidal zones, which act as a nursery (Kuipers and Dapper, 1984, Cattrijsse et al., 1997). They leave the nursery when they reach around 15 mm (Cattrijsse et al., 1997) to 30 mm (Kuipers and Dapper, 1984) total length (TL). Growth is isometric (Oh et al., 1999) and season dependent (Lloyd and Yonge, 1947). Moulting frequency is size, temperature and food availability dependent, with an average intermoult period of two weeks (Evans, 1984, Tiews, 1970). Sexual characters can be noted at 25 mm TL and size at maturity is between 40-60 mm TL (Kuipers and Dapper, 1984, Siegel et al., 2008). The growth rate is sex dependent, with females showing a higher growth rate than males (Oh et al., 1999). Sex-specific size classes can be observed with an equal sex ration until 30 mm TL, followed by higher proportion of males at 30-45 mm TL, after which the female proportion increases to 100% at 60 mm TL (Siegel et al., 2008). *Crangon crangon*

can be considered as a facultative protandric hermaphrodite (with primary females) with a small proportion of the male population possibly being capable of changing sex (Martens and Redant, 1986, Schatte and Saborowski, 2005). First spawning usually occurs at an age of around 21 months (Tiews, 1970) and the length of the spawning season depends on latitude. Spawning occurs all year round at the European continental coast but only during the warmer months at higher latitudes and during the coldest months at lower latitudes (Kuipers and Dapper, 1984, Boddeke, 1982). Although *C. crangon* is a euryhaline species (able to tolerate a wide range of salinity) and can withstand a wide range of temperatures, osmoregulation (especially in males) is inhibited at low temperatures (Lloyd and Yonge, 1947, Viegas et al., 2007). The inshore lower-salinity parts of estuaries are, therefore, avoided during the winter (Henderson and Holmes, 1987, Almut and Bamber, 2013). After winter, females return to the inshore estuaries to brood their young and remain there during the non-reproductive summer-autumn period while males stay in the offshore waters (Henderson and Holmes, 1987, Bamber and Henderson, 1994). The brown shrimp is a short lived species, living up to 2-3 years (sex dependent) and can reach a maximum size of ca. 80 mm TL (Kuipers and Dapper, 1984).

1.2.3. Diet

The brown shrimp can be considered as a trophic opportunist who consumes a wide variety of bottom dwelling organisms including demersal animals, epifauna and infauna (Oh et al., 2001, Tiews, 1970, Evans, 1983). Based on morphological examination of its stomach contents, the trophic ecology of *C. crangon* has been defined in multiple ways including as a trophic generalist (Evans, 1983), carnivorous opportunist (Pihl and Rosenberg, 1984) omnivore (Raffaelli et al., 1989, Tiews, 1970, Lloyd and Yonge, 1947, Ansell et al., 1999) and probable scavenger (Ansell et al., 1999). Large shrimp can prey on commercially important species such as 0-group fish (plaice, dab, sandeel; Oh et al., 2001) and cannibalism can be common in the larger size classes (Pihl and Rosenberg, 1984, Evans, 1983, Evans, 1984). *Crangon crangon* mainly acts as an ambush predator and rarely search actively for prey (Gibson et al., 1995; but see, Tiews, 1970). Feeding mainly takes place during the night (Tiews, 1970, Pihl and Rosenberg, 1984) and food items are detected with the long flagellae of the second antennae which are swept over the surface while the shrimp remains buried (Lloyd and Yonge, 1947, Pinn and Ansell, 1993). Prey items are seized with the aid of the first

and second pairs of pereiopods and ingested in whole (gulping; Tiews, 1970) or macerated to a fine degree (Asahida et al., 1997).

The digestive system of decapod crustaceans is divided into 3 parts: foregut (or stomach), midgut and hind gut. After ingestion, food passes through the oesophagus and enters the stomach (see figure 1.1 for the location of the stomach) which consists of two parts, the cardiac and pyloric chambers, which are surrounded by the hepatopancreas (Felgenhauer, 1992). Consumed sand grains assist in crushing the food in the cardiac part of the stomach (Tiews, 1970). The midgut extends from the foregut through the posterior portion of the hepatopancreas and the abdominal somites to the hindgut. The hindgut is lined with cuticular scales or spines and directs the faeces towards the anus at the base of the telson (Felgenhauer, 1992). The evacuation rate of *C. crangon* is negatively linear related with time and shrimp size and positively with temperature (van der Veer and Bergman, 1987, Pihl and Rosenberg, 1984). Average retention time of food in the stomach is estimated between 2h and 20h (Pihl and Rosenberg, 1984, van der Veer and Bergman, 1987, Feller, 2006). Gut fullness can be influenced by *C. crangon*'s physiological state (lower during pre- and post-moult), reproductive period (lower for females that that are carrying eggs or have advanced ovaries), time of the day and tidal cycle (Oh et al., 2001, Cattrijsse et al., 1997, Pihl and Rosenberg, 1984). Consequently, many shrimp (up to 60% of a catch) can be caught with empty guts (Raffaelli et al., 1989, Oh et al., 2001, Feller, 2006, Pihl and Rosenberg, 1984).

Due to *C. crangon*'s opportunistic feeding behaviour, diet varies with food availability which depends on location, season and substrate (Oh et al., 2001, Pihl and Rosenberg, 1984, Boddeke et al., 1986). Diet also varies with shrimp size; smaller shrimp (< 10 mm Carapace Length, CL) depending more on meiofauna and larger shrimp (> 10 mm CL) more on macrofauna (Oh et al., 2001, Pihl and Rosenberg, 1984). However, due to their relative high abundance, meiofauna prey can still be consumed by larger shrimp (Evans, 1983). Shrimp between 30-45 mm TL show the widest range of food items and males have a more diverse diet than females (Tiews, 1970). Diet overlap with other species is present but is limited due to *C. crangon*'s high variety of prey items (Feller, 2006, Pihl, 1985).

1.2.4. Predation

The wide distribution and high abundance of *C. crangon* makes it an important food item in European waters (Hostens and Mees, 1999, Evans, 1984). Predators of *C. crangon* include a wide range of species such as fish, crustaceans (including cannibalism by large *C. crangon*) and birds (Henderson et al., 1992, Hamerlynck and Cattrijsse, 1994, Hostens and Mees, 1999, Kuipers and Dapper, 1984, Tiews, 1970, Walter and Becker, 1997, Pihl and Rosenberg, 1984). Although *C. crangon* is in some cases only a minor food component (Nierynck and Redant, 1983), it is especially important for the diet of several juvenile fish species (Hamerlynck and Cattrijsse, 1994), due to its year round presence and abundance, in cases where the abundance of other prey items is low (Bamber and Henderson, 1994).

1.2.5. Importance for fisheries

The brown shrimp is an important target species for fisheries with catches up to 35,000 tons in 2011 and more than 500 fishing vessels employed in the North Sea (Campos and van der Veer, 2008, Aviat et al., 2011). Worldwide, *C. crangon* catches represented of 1.3 % of total fisheries in 2005 (Gillett, 2008). In the North Sea, landings show a constant increase since the 1970s with the majority of the shrimp landed in the Netherlands and Germany (ICES, 2013). According to the ICES WKCCM report (2013), the North Sea population consists of one stock which is well mixed and mainly driven by bottom-up processes. There is, however, no efficient management of this stock and there are indications of overfishing (ICES, 2013).

1.3. Animal camouflage and colour changes

The ability of animals to avoid predation by blending with their surroundings can have both immediate and evolutionally consequences, mediated by direct and indirect links between behaviour, physiology and fitness. Their study is, consequentially, of high relevance for a wide range of fundamental and applied questions (Caro et al., 2017, Endler and Mappes, 2017). Animal camouflage is in principle an adaptation to counteract the perception and cognitive mechanisms of other animals and can be achieved in many ways, including background matching, disruptive patterns and countershading (Stevens, 2015, Merilaita et al., 2017). Animal coloration and camouflage are model topics for evolution since they are

easy to study and manipulate experimentally and encompass many essential behaviours and physiological processes, including animal physiology, behavioural ecology and community dynamics (Endler and Mappes, 2017, Nilsson Sköld et al., 2013, Gagliano et al., 2015). Animal colours are under a strong selection pressure and represent often a trade-off between conspicuousness and camouflage (Nilsson Sköld et al., 2013, Gagliano et al., 2015, Stuart-Fox and Moussalli, 2008). Many animals developed the ability to rapidly change colour as a way to deal with this trade-off (e.g. Umbers et al., 2014, Meelkop et al., 2011, Stuart-Fox and Moussalli, 2009, Hanlon, 2007, Nilsson Sköld et al., 2013). These colour changes can also have other ecological and physiological functions including thermoregulation, communication and UV protection (Umbers et al., 2014). The adaptive nature and broad functionality of these colour adaptations can thus provide fundamental insights in how animals adapt to environmental variation (Stevens et al., 2014).

Most studies on colour changes focus on species that show very fast colour changes (within seconds) such as chameleons and cephalopods while most colour-changing species adapt their colour over a longer time (minutes to days; Stevens et al., 2013). Non-molluscan invertebrates such as crustaceans have received relatively little attention (Detto et al., 2008, Umbers et al., 2014). Crypsis is an important part of *C. crangon* ecology. To avoid detection by both predators and prey, it remains buried during most of the day and is able to adapt its colour to match its background (Pinn and Ansell, 1993, Koller, 1927). Colour changes can be achieved morphologically by the anabolism and catabolism of pigments or other colour components (a process which can take days to months) or physiologically by rapid changes (within milliseconds to hours), varying the distribution of pigments (Umbers et al., 2014). Pigmented organelles are located in chromatophores which are specialised cells which can be found just beneath the translucent exoskeleton, deep in muscular tissue and around internal organs (Bauer, 1981). In *C. crangon*, there are five different pigments (black, brown, white, yellow and red; Figure 1.2) which can occur separately in monochromatic chromatophores or in combinations in di- and polychromatic chromatophores (Brown and Wulff, 1941, Koller, 1927, Elofsson and Hallberg, 1973). In contrast to some other organisms (such as *Xenopus laevis;* Hogben and Slome, 1931), chromatophores do not occur individually in *C. crangon* but multiple chromatophores (of similar or different colours) are combined in a structure called the chromatosome (Elofsson and Kauri, 1971). The dispersion and concentration of pigmented organelles within the chromatosomes is the main process

influencing short term colour adaptations in *C. crangon* (Fujii, 2000, Elofsson and Kauri, 1971, Tuma and Gelfand, 1999) and is well-studied at the physiological level (Brown and Wulff, 1941, Fingerman, 1985, Thurman, 1988). Pigment dispersion and concentration can be a direct response (primary) to indent light (Burton, 2010, Brown and Sandeen, 1948) or indirectly (secondary) to neuroendocrine processes caused by light stimuli conveyed to the eyes (Pautsch, 1953). This secondary response is regulated by pigment dispersing and concentrating hormones (see Chang and Thiel, 2015 for the intercellular processes) which mainly originate from the X-organ-sinus-gland complex in the eyestalks of crustaceans (Rao, 2001, Huberman, 2000, Brown and Sandeen, 1948, Perkins and Snook, 1931). Chromatophores are already present at the first larval stage of *C. crangon,* where they show primary responses and function mainly for UV-protection for the essential organs, while background adaptation (by means of secondary responses) becomes functional at later larval stages (Pautsch, 1953). Processes determining colour change in the brown shrimp are at the basic level similar to the ones acting in vertebrates (Fujii, 2000, Elofsson and Kauri, 1971), which makes *C. crangon* a suitable model for colour adaptation studies.

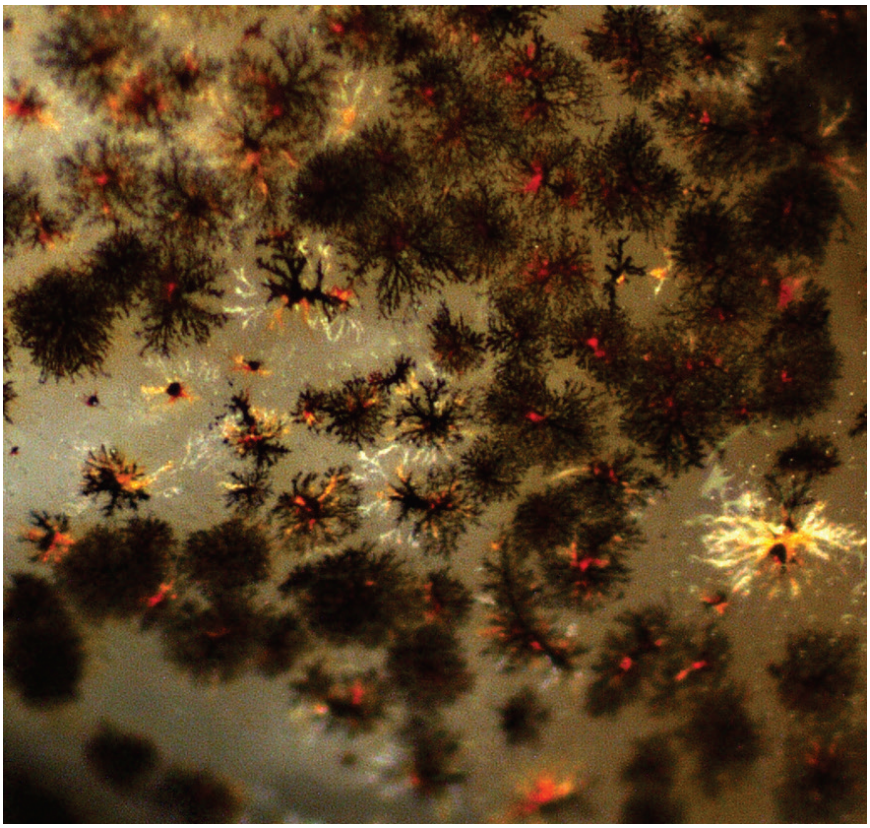

Figure 1.2. Image showing pigments within chromatosomes on the dorsal side of *Crangon crangon*. Image is approx. 0.5 * 0.5 mm.

1.4. Benthic community and trophic metabarcoding

1.4.1. Metabarcoding of environmental and community samples

Assessing the eukaryotic biodiversity of marine systems is a process that traditionally relied on morphology-based taxonomical methods. These methods require expert knowledge, focus on restricted groups of organisms and pose issues with the identification of rare, cryptic or small-sized species and decomposed or digested material (Chariton et al., 2015, Baird and Hajibabaei, 2012, Lejzerowicz et al., 2015). The development of High-Throughput-Sequencing (HTS) techniques, such as metabarcoding (Taberlet et al., 2012a, Hajibabaei et al., 2011), has revolutionized biodiversity assessments by allowing for the simultaneous detection of thousands of species. Metabarcoding refers to the identification of multiple species (or other taxonomic ranks) based on DNA extracted from community (genomic DNA fragments collected from bulk samples of many individuals from a mix of different species; Creer et al., 2016) or environmental samples (i.e. water, soil, faeces; Barnes and Turner, 2016), by means of massive parallel sequencing of polymerase chain reaction (PCR) amplicons generated using primers of varying universality (Barnes and Turner, 2016, Taberlet et al., 2012a, Leray et al., 2015, Pompanon et al., 2012, Kartzinel and Pringle, 2015). Environmental DNA (envDNA) includes all the DNA molecules present in an environmental sample and originated, for example, from living cells, dead tissue, faeces or gut contents, secreted material and extra-cellular DNA. Due to the nature of envDNA samples, these samples can be composed of both community DNA and extra-organismal DNA, which can be present in different physical forms including intramembranous, extramembranous intraorganismal, particulate, adsorbed and free DNA (Barnes and Turner, 2016, Wangensteen and Turon, 2016). Several studies show that a metabarcoding approach can successfully identify small, cryptic and decomposed species with reduced cost and effort compared to traditional methods, and that it provides a more holistic approach to the detection of biodiversity (Chariton et al., 2015, Leray and Knowlton, 2015, Hajibabaei et al., 2011, Lejzerowicz et al., 2015).

Metabarcoding of envDNA and community DNA has been successfully applied for a wide range of research topics including population dynamics (Kartzinel and Pringle, 2015), invasive species (Eichmiller et al., 2016), parasitology (Huver et al., 2015),ecotoxicology (Cornall et al., 2016), and trophic studies and ecological networks (Clare, 2014). Being a

relatively novel technique, the application of metabarcoding still faces challenges which can lead to the inclusion of false positives and negatives, and other biases in the data set. There issues can be roughly divided in (a) errors that are caused by natural processes influencing the degradation speed of DNA and degree of transportation of genetic material from its source (Barnes and Turner, 2016); (b) errors obtained during the PCR-amplification and HTS process, including PCR sequencing errors, tag-switching and marker selection biases (Schnell et al., 2015a, O'Donnell et al., 2016, Alberdi et al., 2018, Taberlet et al., 2012a); and (c) errors related to bioinformatic challenges and taxonomic assignment, such as clustering errors, the inclusion of false positives and negatives and incomplete or incorrect reference databases (Alberdi et al., 2018, Taberlet et al., 2012a, Coissac et al., 2012). Especially the assessment of species abundances based on sequencing reads can be challenging due to variation in primer efficiencies across species and differences in copy number of multi-copy genes across taxa (e.g. ribosomal and mitochondrial genes). Furthermore, uncertainties exist on the effects of organism body size, activity level, metabolism, reproduction on envDNA production and the number of sequence reads detected (Elbrecht and Leese, 2015, Evans et al., 2016b, Barnes and Turner, 2016, Pinol et al., 2015, Prokopowich et al., 2003). Nevertheless, multiple studies, on diverse taxa, showed correlations between relative read abundances and species biomass (e.g., Thomas et al., 2016, Evans et al., 2016b, Guardiola et al., 2015). By using robust repeatable protocols, state of the art techniques and providing transparency about uncertainties, metabarcoding can provide reliable taxonomic information from environmental samples (Barnes and Turner, 2016, Thomas et al., 2016, Lejzerowicz et al., 2015, Taberlet et al., 2012a, Wangensteen and Turon, 2016). Special care should be taken in selecting the right marker gene for the question under research since not all markers provide the same information due to differences in species-resolving power and taxonomic coverage (Creer et al., 2016, Shaw et al., 2016, Clare, 2014). For example, the use of more conservative markers (e.g., ribosomal DNA vs. mitochondrial cytochrome c. oxidase subunit I (COI) region) will result in a potential large taxonomic coverage but will reduce species-level resolution, and smaller amplicons will be able to amplify highly degraded DNA but will result in reduced taxonomic information (Clare, 2014).

1.4.2. Trophic metabarcoding

Trophic interactions form the basis of ecological networks and are essential for ecosystem functioning and services (such as nutrient recycling or the provision of food; Clare, 2014, Evans et al., 2016a, Raffaelli, 2006). The study of these interactions provides essential insights in multiple ecological fields, including ecosystem dynamics, community ecology, food web structure, predator-prey interactions and animal behaviour (Pinol et al., 2014, Leray et al., 2015, Van Tomme et al., 2014). The exact determination of an animal's diet can be very challenging, especially in the case of generalist predators that consume a wide range of taxa, including rare, cryptic and soft bodied species (Feller, 2006, Asahida et al., 1997, Symondson, 2002). Trophic molecular tools allow for a robust analysis of animal scats and dissected or regurgitated stomach/gut contents (Clare, 2014). Trophic metabarcoding methods relaying on versatile primers targeting short hypervariable DNA regions (Leray et al., 2015, Pompanon et al., 2012, Kartzinel and Pringle, 2015) have proved to be highly effective and versatile for the identification of prey remains and can detect a large range of prey items (including small, soft bodied and parasitic organisms) several hours after digestion with a significant higher taxon resolution, precision and speed of analysis than traditional morphological methods (Berry et al., 2015, Casper et al., 2007, Moran et al., 2016, Symondson, 2002). Gut and stomach samples can consist of a mix of community and extra-organismal DNA (including highly digested, fragment and degraded DNA; Creer et al., 2016), incurring a specific set challenges in comparison to other envDNA samples (Clare, 2014). First, quantitative descriptions of food intake based on gut contents might be biased by a number of factors (many of which also bias traditional diet qualifications; Casper et al., 2007) including variation of the consumed prey size, presence of hard carapace/shell material, amount of soft tissue ingested and species-specific digestion and degradation rates (Deagle et al., 2010, Kartzinel and Pringle, 2015, Clare, 2014). Though studies show that DNA extracted from animal scats can provide semi-quantitative information (Deagle et al., 2005, Deagle et al., 2010, Thomas et al., 2016), the reliability of relative read abundances of stomach/gut contents is still not fully clear. Nevertheless, several studies show comparable results based on relative abundance data and presence-absence data obtained from stomach/gut samples (Albaina et al., 2016, Ray et al., 2016). Second, metabarcoding is not able to detect secondary predation, cannibalism and distinguish different ontogenetic stages (Deagle et al., 2010, Kartzinel and Pringle, 2015, Berry et al., 2015, Casper et al., 2007). The bias caused by secondary predation in metabarcoding diet studies is not fully understood,

but likely depends on the trophic level of the prey items (Leray et al., 2015, Kartzinel and Pringle, 2015, Berry et al., 2015). It can, nevertheless, be assumed that the amount of DNA from secondary prey should be minor and highly digested (so lower detection rate) than from primary prey (Leray et al., 2015).

Overall, metabarcoding is considered to be a very effective technique in describing the full diet of predators and resolving complex species interactions and food webs (Pinol et al., 2014, Leray et al., 2015, Burgar et al., 2014). Trophic metabarcoding could be used as a tool in conservation biology to identify key food web links, study ecological networks and detect vulnerable or invasive species (Clare, 2014). The application of this novel molecular technique to assess the diet of key-stone species such as *C. crangon* can provide information of the overall stability of the whole system and be of great value for ecosystem management, especially in complex dynamic systems such as estuaries.

1.5. Aims and objectives

This thesis focusses on the anti-predator behaviour and opportunistic diet of the brown shrimp *Crangon crangon* L., which are main components of this shrimp's ecological success and pivotal role in European coastal and estuarine ecosystems. The brown shrimp is widely distributed in European estuaries and is well-known for its colour changing abilities and central role in the food web of soft-bottom habitats (Evans, 1984, Koller, 1927), yet little information is available on the adaptive and variable nature of its colour changing behaviour and prey selection. A multidisciplinary approach, encompassing novel behavioural and molecular tools, was applied to address the following objectives:

- Quantify colour change in *C. crangon* (**chapter 2**) and examine the effects of spatial and temporal environmental heterogeneity of the soft-bottom habitat on its colour changing ability, using lab experiments (**chapter 3**)
- Assess the application of metabarcoding to describe the diet of *C. crangon* and reveal variations in its trophic ecological function on a European scale (**chapter 4**)
- Evaluate the suitability of trophic metabarcoding of *C. crangon* stomach contents as an environmental assessment tool, from an ecotoxicological perspective (**chapter 5**) and as a novel biodiversity monitoring tool to monitor fish presence (**chapter 6**)

Chapter 2.

Quantifying pigment cover to assess variation in animal colouration

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2.1. Abstract

The study of animal colouration addresses fundamental and applied aspects relevant to a wide range of fields, including behavioural ecology, environmental adaptation and visual ecology. Although a variety of methods are available to measure animal colours, only few focus on chromatophores (specialized cells containing pigments) and pigment migration. Here, I illustrate a freely available and user friendly method to quantify pigment cover (PiC) with high precision and low effort using digital images, where the foreground (i.e., pigments in chromatophores) can be detected and separated from the background. Images of the brown shrimp, *Crangon crangon* were used to compare PiC with the traditional Chromatophore Index (CI). Results indicate that PiC outcompetes CI for pigment detection and transparency measures in terms of speed, accuracy and precision. The proposed methodology provides researchers with a useful tool to answer essential physiological, behavioural and evolutionary questions on animal colouration in a wide range of species.

2.2. Introduction

The study of animal colouration and colour patterns is essential to gather a better understanding on how animals visually communicate and how they can match different substrates. Furthermore, this type of studies provides important insights on how predation avoidance due to camouflage can drive inter- and intra-specific variation, and how colouration and visual perception are connected (e.g. Stevens et al., 2013). A wide range of methods has been developed to measure animal colouration, which can be roughly divided in three categories: (1) spectral quantification of colouration and animal vision (Stevens et al., 2009, White et al., 2015); (2) assessment of colour patterns (Holmes, 1940, Hanlon, 2007, Taylor et al., 2013, Merilaita and Dimitrova, 2014); (3) analysis of chromatophores and pigment migration (Koller, 1927, Perkins and Snook, 1931, Darnell, 2012). The last method has been used mainly to study animal colour changes (Perkins and Snook, 1931, Nilsson Sköld et al., 2013, Umbers et al., 2014).

Chromatophores are specialised cells containing pigmented organelles and can be located in the dermis, epidermis, beneath a translucent exoskeleton, deep in muscular tissue or around internal organs (Elofsson and Kauri, 1971, Bauer, 1981, Fujii, 2000). In crustaceans, multiple tightly bound chromatophores (of similar or different colours) are combined in a structure called chromatosome (Elofsson and Kauri, 1971, McNamara, 1981). Many animals can regulate their colour by the dispersal and concentration of pigments within chromatophores (e.g. Meelkop et al., 2011, Umbers et al., 2014): colour can be changed in a period of days to months through anabolism and catabolism of pigments and cells (morphological colour change) or within milliseconds to hours via the migration of pigments within chromatophores (physiological colour change; Umbers et al., 2014). The concentration or dispersion of pigments reduces or increases their visibility, since less or more surface area is covered by them, respectively (Smith, 1938, Peter et al., 2011). Hogben and Slome (1931) described changes in the pigment distribution in the frog *Xenopus laevis* by classifying chromatophores in 5 classes (Figure 2.1), applying a Melanophore Index for melanophores (also more generally called Chromatophore Index (CI) for chromatophores containing pigments other than melanin; Hogben and Slome, 1931, Auerswald et al., 2008). Although this method has been extensively used (see Table 2.1 for some recent examples), concerns have been raised about its degree of subjectivity, statistical validity and labour intensiveness (Parker, 1943, Flores and Chien, 2011). Here, I describe a new method, Pigment Cover (PiC),

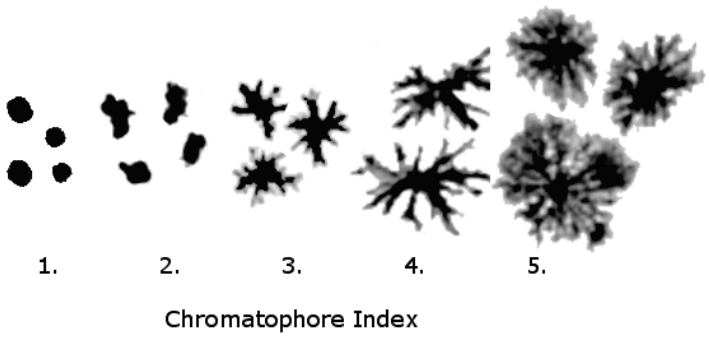

Figure 2.1. Stylised representation of the different classes of pigment dispersion in *Crangon crangon* chromatophores. Classification is based on the Melanophore Index of Hogben and Slome (1931).

Table 2.1. Selected publications applying the Melanophore Index (MI) of Hogben and Slome (1931).

Group	Species	Area of interest	Topic	Method	Source
Amphibian	*Bufo melanostictus*	Dorsal skin	Drug development	MI*	(Ali and Naaz, 2015)
	Hoplobatrachus tigerinus	Isolated dorsal skin cell	Physiology	MI*	(Ali et al., 2012)
	Rana catesbeiana	Dorsal skin	Endocrinology	MI	(Gao et al., 2015)
	Taricha granulosa	Larva	UV-Protection	MI	(Belden and Blaustein, 2002)
	Ambystoma gracile	Larva	UV-Protection	MI	(Belden and Blaustein, 2002)
	Ambystoma macrodactylum	Larva	UV-Protection	MI	(Belden and Blaustein, 2002)
	Xenopus laevis	Larva	Developmental biology	MI	(Eagleson et al., 2012, Eagleson et al., 2010)
Crustacean	*Chasmagnathus granulata*	Maxilliped's meropodit	UV-Protection	CI	(Gouveia et al., 2004)
	Palaemonetes argentinus	Dorsal abdomen	UV-Protection	CI	(Gouveia et al., 2004)
	Eurydice pulchra	Not specified	Endocrinology	CI*	(Wilcockson et al., 2011)
	Palaemon pacificus	Dorsal abdomen	Endocrinology	CI	(Meelkop et al., 2012, Marco and Gäde, 2010)
Reptile	*Hemidactylus flaviviridis*	Dorsal skin	Drug development	MI*	(Ali and Meitei, 2011)
Teleost	*Ctenopharyngodon idellus*	Scale	Physiology	MI	(Jiang and Wong, 2013)
	Danio rerio	Scale & Embryo	Physiology	MI	(Xu and Xie, 2011)
	Oncorhynchus mykiss	Scale	Ecotoxicology	MI	(Lennquist et al., 2010)
	Verasper moseri	Base of caudal fin	Developmental biology	MI	(Yoshikawa et al., 2013)

*Modified Index; MI: Melanophore Index (pigment is melanin); CI: Chromatophore Index (pigment is not melanin).

to assess the degree of pigment dispersion within chromatophores (or chromatosomes) by measuring the coverage of pigments in defined areas of an animal body, thus allowing us to evaluate colour variations in a quantitative way. The objective of this study is to demonstrate the use and versatility of PiC and compare it to the established CI. To achieve this, both PiC and CI were applied to a database of pictures of the brown shrimp, *Crangon crangon* (L.), a crustacean characterized by good background-matching abilities (Koller, 1927).

2.3. Material and methods

2.3.1. Protocol to measure pigment cover

2.3.1.1. Image acquisition

Measurements on animal colour or pigment migration are usually performed on a specific body region rather than the whole animal (Brown and Sandeen, 1948, Darnell, 2012, Stevens et al., 2013). In some cases, e.g., fish scales (Xu and Xie, 2011), the area of interest can be separated from the animal prior to image acquisition, reducing the effects of animal stress on the colour (Nguyen et al., 2006). The specimen should be placed and photographed on a uniform surface (Figure 2.2A). Contrast between background and pigments should be as high as possible; overlap with underlying organs should be avoided, if possible (Flores and Chien, 2011). The magnification should be high enough to distinguish individual chromatosomes. If multiple pigments are studied, the collection of multiple images of the same area on different backgrounds might be necessary (see below). To optimize image acquisition, illumination within an image should be uniform and shadows or reflection of light should be avoided. Light conditions are, nevertheless, less constricted than in other methods (Appendix 1.1; Stevens et al., 2007, White et al., 2015) and colour charts are not required (they can vary in quality and applicability; Stevens et al., 2009, White et al., 2015). Still, standardisation of lighting conditions and camera settings will significantly reduce the use of manual adaptations during image analysis (see Stevens et al. (2007) for more information on the standardisation of digital images). In digital photography, images are commonly displayed in a non-linear standard default colour space (sRGB). PiC can be applied to these standard images. For more rigorous and objective image analyses, if needed linear images are often required. If this is the case, sRGB images can be converted to the CIELAB colour space using the “Color Space Converter” plugin of ImageJ (https://imagej.nih.gov/ij/plugins/color-space-converter.html). A normalisation step is advised to slightly enhance the contrast within the images by using the *Enhance Contrast* command of ImageJ. A slight over-saturation of 1% is advised for improved visual evaluation (Brocher, 2014).

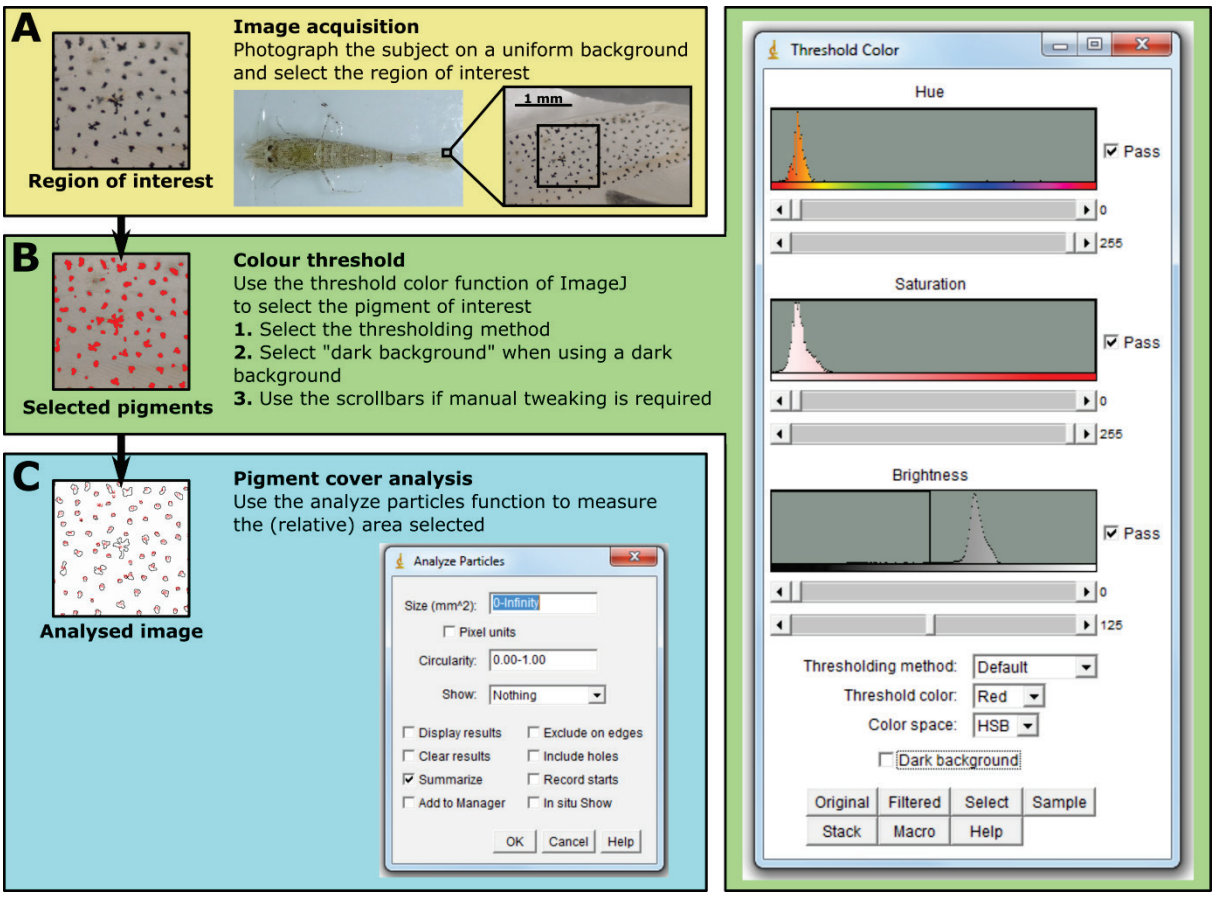

Figure 2.2. Protocol for pigment cover measurements. This diagram outlines the steps to be performed in ImageJ to determine pigment cover (PiC). See text for details.

2.3.1.2. Colour threshold

Pigment cover image analysis can be performed with any graphic editor able to perform image segmentation (partitioning an image into sets of pixels) by means of thresholding. Image segmentation by semi-automatic thresholding is an established method which has been used in a range of biological studies, including crop root length (Tajima and Kato, 2011), plant signals (Swanson et al., 2006) and cell counts (Drury et al., 2011), but not specifically on pigment coverage. The methodology described in this section is tailored to the freely available java-based imaging program (ImageJ 1.48v http://imagej.nih.gov/ij/; Schneider et al., 2012) because of its ease of use and efficacy, but could easily be adapted to other graphic software.

Images need to be cropped to the region of interest and segmented to differentiate foreground (the pigments under study) and background (Hartig, 2001). In ImageJ, sRGB image segmentation is achieved with the *Color Threshold* function (Figure 2.2B), which segments 24-bit RGB images based on pixel values (see the ImageJ user guide; Ferreira and

Rasband, 2012). A range of automatic thresholding algorithms is available in ImageJ. These algorithms perform differently depending on the distribution of pixel values in the image and the most suitable thresholding algorithm should be selected prior to analysis (Tajima and Kato, 2011, Brocher, 2014), e.g., using the *Threshold Check* macro of the BioVoxxel toolbox (http://www.biovoxxel.de/development/, http://fiji.sc/BioVoxxel_Toolbox#Threshold_Check). The sensitivity of the threshold function can be manually adapted using the *Saturation* and *Brightness* scroll bar in the colour threshold settings window (Figure 2.2B) until all the area covered by the pigment(s) of interest is selected (Drury et al., 2011, Jensen, 2013). Manual alteration of the thresholding level reduces, however, the objectivity of the analysis and should be avoided as much as possible. Specific pigments can also be selected by adapting the *Hue* scroll bar (Figure 2.2B) to the required hue values (Swanson et al., 2006). For transparency measurements, the *Hue* scroll bar should be used to select the background colour to ensure that only the transparent area is selected (the background will be visible through transparent tissue) and all pigments are ignored. In cases where only one channel of the image is analysed (e.g. CIELAB's L channel or grayscale images), ImageJ's *Threshold* function can be used in similar fashion as the C*olor Threshold* function.

2.3.1.3. Pigment cover analysis

The area of the selected pigment(s) can be calculated with the *Analyze Particles* command (Figure 2.2C) which measures "particles" (separate shaped objects) in an image after thresholding by scanning the image and outlining the edge of objects found has been performed (Ferreira and Rasband, 2012, Papadopulos et al., 2007).

2.3.2. Case study

2.3.2.1. Dark and light pigment measurements and transparency

Five specimens of *C. crangon* were selected based on visual differences in colour. Their right exopod (the external branch of their tail fan) was photographed under a stereo microscope (Leica S6D) with a Leica DFC295 camera. The tail fan is the most suitable body area of caridean shrimp to be used for monitoring chromatic parameters because: (1) it is very flat, (2) has no underlying organs or tissue (and is thus highly transparent) and (3) it can be photographed while causing minimal stress to the animal (Flores and Chien, 2011, Brown

and Wulff, 1941). Artificial illumination was provided by two led spotlights (JANSJÖ; 88 lm; 3000 Kelvin) positioned at either side of the microscope. The white balance was adjusted prior to image collection and allowed the exposure time to be automatically adapted. Images were collected in sequence, on four differently coloured backgrounds (Figure 2.3): white for the measurement of dark-coloured (black and sepia-brown) pigments; black for light-coloured (white and yellow) pigments and green and blue for transparency measurements. Green and blue hues do not occur naturally in *C. crangon* (Koller, 1927, Brown and Wulff, 1941) and are therefore suitable for transparency measurements (both colours were used in order to test which one performs better). To avoid adaptation to the background during the measurements, shrimp were kept for a very short duration only (less than one minute) on each background. Images were saved in uncompressed TIFF format (RGB), cropped to 1mm^2 and analysed following the protocol described above, using the default thresholding method, based on the IsoData algorithm (Ridler and Calvard, 1978, Landini, 2015). Manual adaptation was applied if needed. I selected the default thresholding algorithm for this experiment since it performed best for the variety of features (dark pigment, light pigment, transparency) tested. For the same photos, we determined the CI, in accordance to the method of Hogben and Slome (1931), by classifying all chromatosomes in the selected area individually and averaging their values (see figure 2.1 for reference).

2.3.2.2. Dark Pigment Cover and Chromatophore Index comparison

Fifty sRGB images of *C. crangon* (Figure 2.4; obtained from 36 individual shrimp) were selected to represent the range of colouration shown by shrimp (lighter or darker, depending on the substrate where animals were kept). Images varying in properties such as illumination and picture quality were selected to test the robustness of the methodology used. All images were obtained on a white background and cropped to 1 mm^2 in the centre of the exopod. Images were analysed for dark pigments, which are the most abundant and evident pigments responsible for dark colouration (Koller, 1927, Brown and Wulff, 1941). Three observers analysed the images with the PiC and CI methods, in random order. Prior to analysis, the optimal thresholding algorithm was determined by applying a threshold check (BioVoxxel toolbox) to a sub-selection of 13 images. Based on the average score of these images, the MaxEntropy algorithm (Kapur et al., 1985) was selected for all images. Manual adaptation was applied as little as possible (on average on 23% of the images, depending on

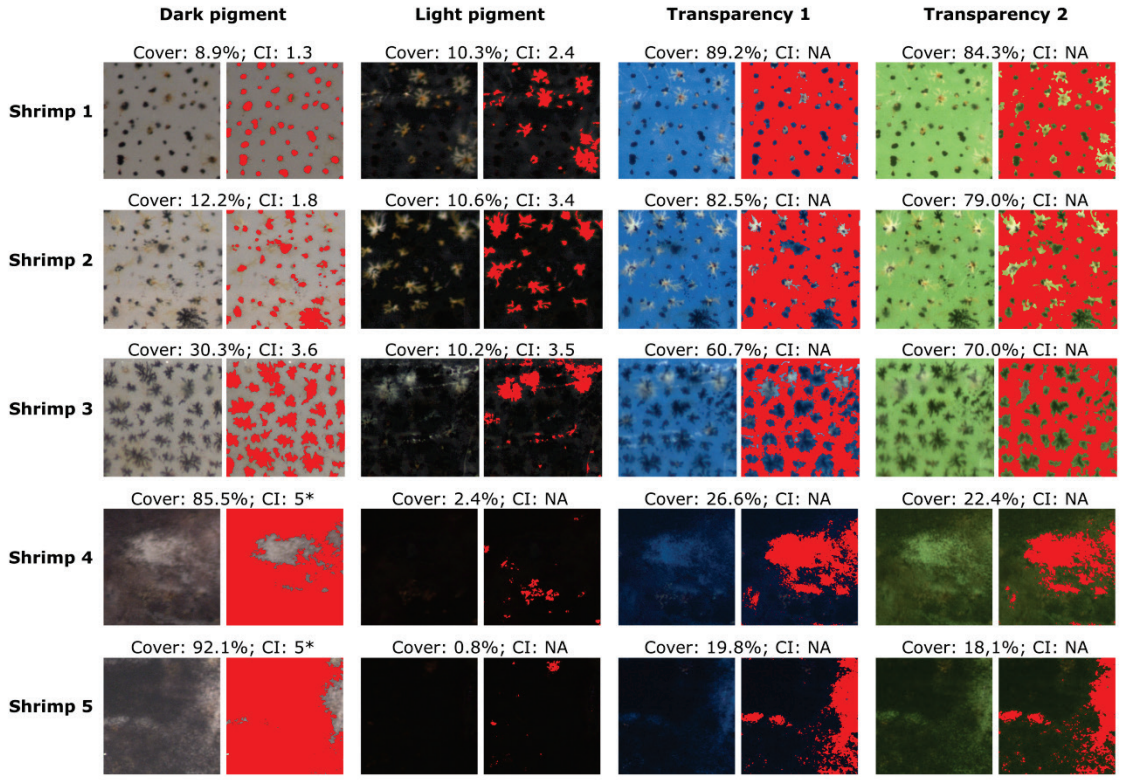

Figure 2.3. Pigment and transparency values for cover (%) and Chromatophore Index (CI) for five shrimp (*Crangon crangon*) on different backgrounds. For each specimen, the right exopod was photographed, always in the same exact position and then the image cropped in the centre (selecting 1mm^2). Red areas represent the area selected by the PiC method. NA: CI cannot be calculated. *CI is an estimate.

the observer). To test the effect of image linearization and normalisation, the 50 sRGB images were transformed to the CIELAB colour space and the L channel was normalised prior to PiC determination. The MaxEntropy thresholding algorithm was applied and, in this case, no manual adaptation was allowed to eliminate the need for subjective input. PiC values of the sRGB (averaged over the observers) and linearized/normalised images were compared using linear regression.

2.3.2.3. Data analyses

Inter-observer variation for both dark pigment cover and CI was tested with the Friedman's test. This statistical test was selected because of the non-normal distributed nature of both proportions and ordinal data and the fact that each image was tested repeatedly. Both PiC (percentage of pigment cover transformed to fraction) and CI results were averaged between observers and a beta regression (betareg R package; Cribari-Neto and Zeileis, 2010) was used to compare the methods. This specific analysis can also be important to predict the

results from one method (PiC) when having information from the other (CI). Different link functions (log, log-log and logit) were compared based on AIC. Beta regression is considered a suitable test for non-parametric and bounded data such as proportions (Cribari-Neto and Zeileis, 2010). Data analyses were performed with R statistical software v.3.1.2 and IBM SPSS statistics v. 20.

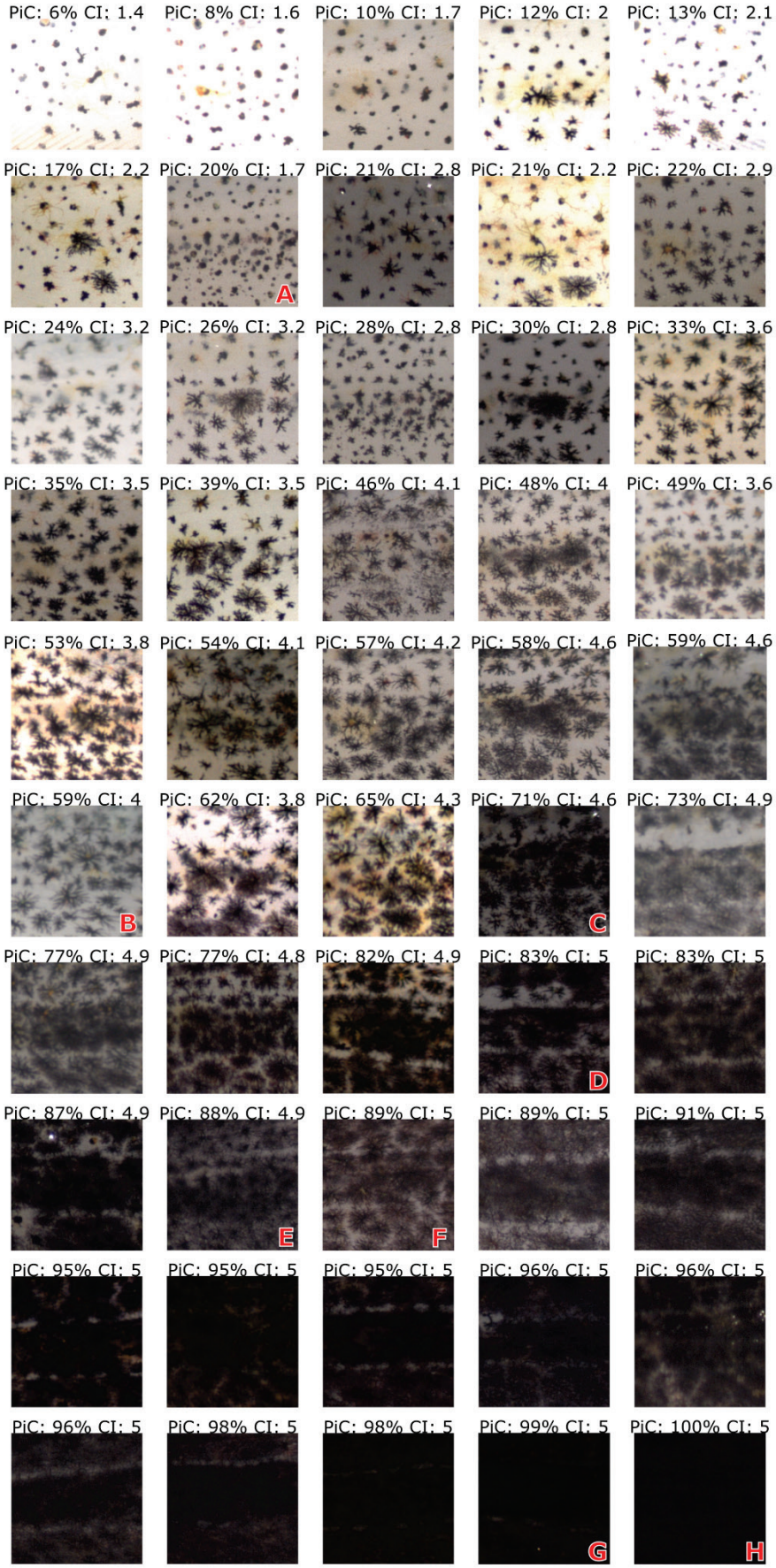

Figure 2.4. Percentage dark pigment cover (PiC) and Chromatophore Index (CI) of 50 images (1mm2) of *Crangon crangon*'s exopods. The images show different levels of chromatosome dispersion and represent the range of coloration exhibited by the animals. See text for information on capital letters.

2.4. Results

2.4.1. Dark and light pigment measurements and transparency

For the five specimens analysed, dark PiC values ranged from 8.9% to 92.1% and light PiC values from 0.8% to 10.6% (Figure 2.3). Transparency measurement ranged from 19.8% to 89.2% on a blue background and from 18.1% to 84.3% on a green background (mean difference ± s.d.: 1±5.9%) and did not significantly differ between the background colours (Wilcoxon Signed Ranks Test: N = 5, Z = -0.674, *P* = 0.500). CI, by definition, cannot be calculated for transparency (Figure 2.3). When dark pigments were predominant (e.g., shrimp 4 and 5 in Figure 2.3), the CI of light pigments could not be calculated, as it was impossible to distinguish their shape. Furthermore, the high overlap of dark chromatosomes made it impossible to count the number of chromatosomes to calculate the mean dark CI. In these cases, the CI was estimated as 5, the maximum index value.

2.4.2. Dark pigment cover and chromatophore index comparison

PiC and CI for all 50 images were calculated (Figure 2.4). Dark PiC showed a strong exponential relationship with CI (Figure 2.5) and the beta regression confirmed a significant relationship between PiC and CI (coefficient ± s.e.m.: 0.659 ± 0.034; *P* <0.0001) with a Pseudo R^2 value of 0.95. The equation to estimate PiC from a known CI value was modelled as:

$$\text{Ln (predicted PiC)} = -3.362 + 0.659 * \text{CI}$$

The equation is only valid for: $1 \leq CI \leq 5$ and $0 \leq PiC \leq 1$. According to AIC values, the log link function (AIC: -127) provided a better fit than models with a logit (AIC: -82) or log-log (AIC: -66) link function. In half of the images the observers were not able to provide a reliable count of the maximum dispersed chromatosomes, necessary to calculate the CI, due to a high level of overlap between the chromatosomes. Above 63 ± 9% PiC, individual chromatosomes overlapped resulting in unreliable CI estimates; above 80 ± 9% PiC it was not possible to detect any difference based on CI since all chromatosomes were in the highest category (CI = 5). No problems were encountered during the estimation of pigment cover, including the darkest images. The observers spent on average 75 ± 5 min calculating the CI and only 18 ± 9 min determining PiC. Results differed significantly among observers for both methods (Friedman's Test: CI: N = 50, d.f. = 2, X^2 = 11.09, *P* = 0.04; PiC: N = 50, d.f. = 2, X^2 =

18.67, $P < 0.001$), with an average relative standard deviation over all images of 3% for CI and 6% for PiC. Individual regression parameters were similar among the observers (Appendix 2.2) and the majority of the variation in the PiC estimates was caused by one observer who relied on manual adaptation (N = 23) much more than the other observers (N = 6 and N = 5). Linear regression estimates of PiC values of sRGB versus linearized/normalised images showed that both methods produce concordant results (Appendix 2.3; d.f. = 45, $R^2 = 0.995$, $P < 0.001$, slope = 0.948), indicating that the use of sRGB images does not produce significant systematic errors in this case.

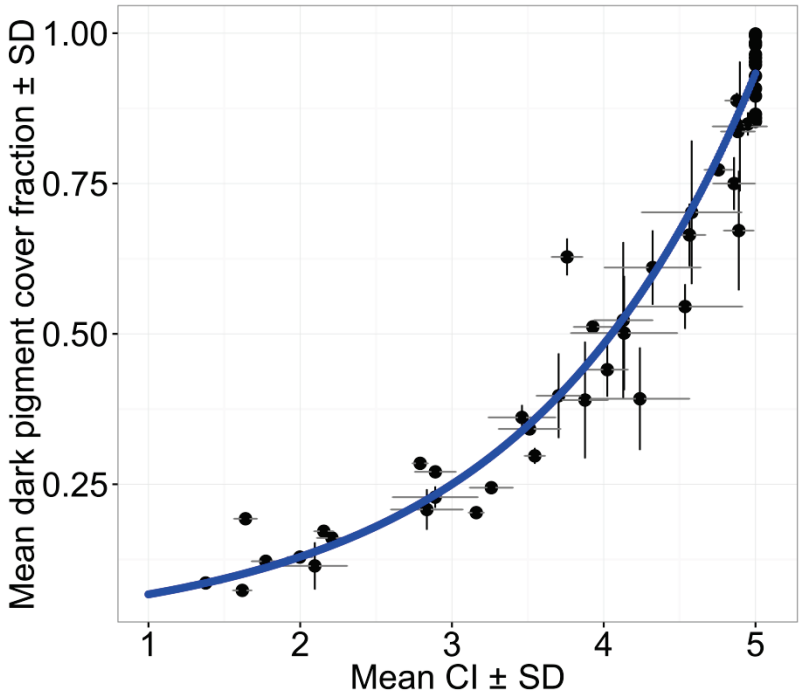

Figure 2.5. Relationship between Chromatophore Index (CI) and dark pigment cover fraction. Measurements were performed on 50 images of *Crangon crangon* (see Figure 2.4). Mean values and s.d. for the readings of three observers are given per image. The solid line shows the beta regression fit (with log link function).

2.5. Discussion

Animal colouration can be assessed by determining pigment dispersion in individual chromatophores or in multicellular chromatosomes (e.g. Auerswald et al., 2008, Peter et al., 2011). The traditional and widely used Chromatophore Index (Hogben and Slome, 1931) classifies individual chromatophores or chromatosomes based on their physiological state, indexing their extent of dispersion. As a result, the CI does not provide information on their morphological state (abundance of pigments). Animals with widely spaced, but fully dispersed, chromatosomes (Figure 2.4D) have, consequently, the same maximum index (CI = 5) as animals with a high abundance and overlap of chromatosomes (Figure 2.4H), even though the difference in darkness is visually apparent. This issue has already been considered by Parker (1943) who observed catfish with clear differences in darkness, not distinguishable by the values of CI (all falling in the maximum category). Methods relying on the measurement of the diameter of the chromatosomes (McNamara and Ribeiro, 2000, Peter et al., 2011) have the same problem, since they also omit morphological variation (Parker, 1943). PiC combines both information on the distribution and abundance of pigments and is, therefore, able to distinguish physiological differences within the same animal (Figure 2.4 A vs. E) and morphological differences between animals with the same physiological chromatosome state (Figure 2.4 F vs. G), even in very dark animals (PiC > 80%). The comparison between PiC and CI shows the range where it is possible to transform the values from one method to the other and where PiC is more precise than CI. The logarithmic relationship indicates that the more dispersed the pigments are, the more effective the PiC is in detecting small differences between images compared to the CI. Thresholding methods are considered a more reliable tool for image analysis than human judgement (Drury et al., 2011). Nevertheless, the accuracy and objectivity of PiC is influenced by the amount of manual adaptation applied. The database used during this study consisted of images taken under a variety of lighting conditions to show the wide applicability of PiC. However, automatic thresholding algorithms work best with images taken with identical lighting conditions and camera settings. Manual adaptation of the threshold values, required in cases where the image quality was not optimal (e.g. Figure 2.4 B & C), resulted in increased observer variation and subjectivity. In studies where standardisation of the images is not possible, extra care should be taken to ensure the objectivity of the study (e.g., observers being made blind to the treatments; between-observer repeatability analysis). These

considerations should also be taken into account for the CI. The CI is furthermore less precise in darker animals, and it takes up to 4 times longer than PiC. This difference in analysis speed is due to the fact that the CI can only be determined by the manual classification of every single chromatosome in the image. Moreover, PiC allows testing for transparency, which is important in studies of colour change (Auerswald et al., 2008, Nilsson Sköld et al., 2010).

Digital photography is a popular technique in animal colouration research due to its availability, speed, relative low price and ease of data acquisition (Stevens and Merilaita, 2009, Stevens et al., 2007, Troscianko and Stevens, 2015). Although there are issues with the use of digital images in animal colour studies (Stevens et al., 2007), most of these relate to the control for variation in lightning conditions and the conversion of images to animal vision systems (Stevens et al., 2007, Troscianko and Stevens, 2015). Most cameras produce non-linear images (e.g., sRGB) which generally over- or under-estimate light values and rigorous image analysis methods should include linearization and normalisation of these images (Stevens et al., 2007, Troscianko and Stevens, 2015). PiC focusses on *a priori* specified pigments and does not rely on the exact colour or observer's vision system. In this method, the difference between foreground and background pixels in an image is more important than the exact colour, thus stable lighting conditions are less relevant for PiC than for methods requiring linearized images. Studies that analyse chromatophores and pigment migration (see table 2.1 for examples) usually focus on a limited number of pigments, in high contrast with the background. In these types of studies, PiC can be used also with sRGB images (as shown by the concordant PiC values of sRGB and linearized images reported above) as long as the users are aware of the limitations of the use of non-linear images. In cases where a more precise, objective and rigours determination of animal colour is required, image normalization and standardisation can be performed prior to PiC determination. Standardization of lighting conditions and camera settings is also advised in these cases. Besides being less constrained regarding lighting conditions, PiC is also easy to use and fast in the analysis of large surfaces (opposed to spectrometry; White et al., 2015).

The study of animal colouration is a broad field of investigation encompassing molecular, cellular, physiological, behavioural and evolutionary questions (Stevens et al., 2013, Umbers et al., 2014). The proposed methodology combines the advantages of digital image acquisition with the power of a free open-source program. PiC is simple to use, can be easily

employed also for educational purposes (see: Heggland et al., 2000) and can be applied in any system where rapid colour change is determined by pigment migration in chromatophores. The brown shrimp's chromatosomes system is a widely applicable model since its physiological factors are well studied and its pigment system is complex and essentially similar to those of vertebrates (Keeble and Gamble, 1904, Koller, 1927, Elofsson and Hallberg, 1973, Elofsson and Kauri, 1971, Rao, 2001). The proposed method will thus be a useful tool in future investigations on animal colouration as a fast and effective proxy for the interpretation of complex and dynamic biological systems in a wide range of species.

Chapter 3.

Background matching in the brown shrimp *Crangon crangon*: adaptive camouflage and behavioural-plasticity

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3.1. Abstract

A combination of burrowing behaviour and very efficient background matching makes the brown shrimp *Crangon crangon* almost invisible to potential predators and prey. This raises questions as to how shrimp succeed in concealing themselves in the heterogeneous and dynamic estuarine habitats they inhabit and what type of environmental variables and behavioural factors affect their colour change abilities. Using a series of behavioural experiments, we show that the brown shrimp is capable of repeated fast colour adaptations (20% change in dark pigment cover within one hour) and that its background matching ability is mainly influenced by illumination and sediment colour. Novel insights are provided on the occurrence of non-adaptive (possibly stress) responses to background changes after long-time exposure to a constant background colour or during unfavourable conditions for burying. Shrimp showed high levels of intra- and inter-individual variation, demonstrating a complex balance between behavioural-plasticity and environmental adaptation. As such, the study of crustacean colour changes represents a valuable opportunity to investigate colour adaptations in dynamic habitats and can help us to identify the major environmental and behavioural factors influencing the evolution of animal background matching.

3.2. Introduction

The requirement of prey to avoid detection by their predators has led to the evolution of a wide range of strategies for animals to blend into their environment. Among crustaceans, some species are transparent (Johnsen, 2001) and thus almost invisible, others allow the growth of epiphytes on their carapace (Ruxton and Stevens, 2015) or match their colour with the background, to conceal themselves (Duarte et al., 2017, Merilaita et al., 2017). Indeed, the ability to rapidly change colour is a common strategy employed by many animals to tune and adjust their camouflage abilities to heterogeneous environments (e.g. Umbers et al., 2014, Meelkop et al., 2011, Stuart-Fox and Moussalli, 2009, Hanlon, 2007, Nilsson Sköld et al., 2013), as well as to communicate with conspecifics, thermoregulate and gather protection from ultraviolet (UV) light (Darnell, 2012, Fuhrmann et al., 2011, Umbers et al., 2014). Colour adaptations represent a complex and multifaceted topic which connects environmental factors, animal behaviour, visual perception and cell physiology (Nilsson Sköld et al., 2013, Gagliano et al., 2015). The assessment of how these factors are interlinked (Endler, 1995) and contribute to the animal's camouflage strategy is essential to understand the evolution of colour change and background matching in animals.

Crustaceans can change colour morphologically by the anabolism and catabolism of colour components (e.g. pigments; in days to months) or physiologically by rapid changes (in milliseconds to hours) in the distribution of pigments, microstructures or by changing the refractive index of layers in their integument (Umbers et al., 2014). Physiological colour changes by pigment migration within chromatophores (specialised cells containing pigmented organelles which can be dispersed or concentrated; Figure 3.1; Fujii, 2000, Elofsson and Kauri, 1971, Tuma and Gelfand, 1999) are found in a variety of taxa (Umbers et al., 2014) and are well-studied at the physiological level (Brown and Wulff, 1941, Fingerman, 1985, Thurman, 1988). Evidence on how colour changes fit in an ecological or evolutionary context is however still limited (Stuart-Fox and Moussalli, 2009, Umbers et al., 2014, Nilsson Sköld et al., 2013).

Many crustaceans live in heterogeneous and dynamic intertidal systems in which biotic and abiotic factors vary over multiple spatial and temporal scales. A variety of these factors have the potential to influence camouflage strategies: the evolution and adaptive function of crustacean background matching should be partly driven by environmental variability (Nilsson Sköld et al., 2013, Darnell, 2012, Fuhrmann et al., 2011, Umbers et al., 2014). Having

a degree of flexibility in camouflage strategies is thus advantageous in these heterogeneous environments due to a continuous trade-off between conspicuousness and concealment (Stevens et al., 2014, Nilsson Sköld et al., 2013, Gagliano et al., 2015, Stuart-Fox and Moussalli, 2008). Several environmental factors, such as temperature, tide levels, background, circadian rhythm and predation (e.g. Umbers et al., 2014, Brown and Sandeen, 1948, Detto et al., 2008, Smith, 1930, Palma and Steneck, 2001) have been studied in a few crustacean species including fiddler crabs (e.g. Hemmi et al., 2006, Brown and Sandeen, 1948), crabs (Todd et al., 2012, Stevens et al., 2013) and the shrimp *Hippolyte obliquimanus* (Duarte et al., 2016). Integrated approaches testing multiple environmental variables acting on other crustacean species are, however, rare.

Crangon crangon L. (Decapoda: Caridea) is a key species in European waters and an important target for fisheries (Cattrijsse et al., 1997, Tiews, 1970, Evans, 1984, Campos and van der Veer, 2008, Aviat et al., 2011). Colour change is observed in the adults of this benthic shrimp (Figure 3.1), which is surprising considering its lifestyle, with animals often found buried into the sediment, only eyes and antennae visible (Pinn and Ansell, 1993). Its chromatophore system is well studied and was one of the earliest models of endocrine regulation of chromatophores (Elofsson and Hallberg, 1973, Rao, 2001, Elofsson and Kauri, 1971, Keeble and Gamble, 1904, Koller, 1927). Chromatophores are not individually distinguishable in *C. crangon*, but are combined (with multiple chromatophores of similar or dissimilar pigments) in a structure called the chromatosome (Elofsson and Kauri, 1971). Chromatosomes are also found in transparent larvae (Figure 3.1B). Larvae are pelagic and after five weeks in the water column, post-larvae settle in shallow waters in estuaries (Cattrijsse et al., 1997, Campos and van der Veer, 2008). Chromatosomes in the transparent larvae are probably used for thermoregulation and UV protection at this stage (see below).

Seasonal migration to and from offshore mating areas characterize *C. crangon*'s life cycle (Cattrijsse et al., 1997, Campos and van der Veer, 2008). Parallel to this broad seasonal and spatial variation, juveniles and adults experience local smaller scale variations in estuarine environment. Indeed, habitat characteristics such as illumination, presence or absence of vegetation, sediment colour and sediment composition vary frequently and sometimes unpredictably over space and time in intertidal areas. Biodiversity is increased along a "gradient of structural complexity"(Gross et al., 2017) due to habitat selectivity or ability of organisms to locally adapt to patchy/mosaic habitats.

Figure 3.1. *Crangon crangon* colour. Chromatosome pigments in exopod (part of the tail fan) of *Crangon crangon* (A); transparent larva of *C. crangon* with visible chromatosomes (B); physiological variation in *C. crangon* colouration in response to different coloured background: the same individual was photographed after having been placed on white (C) or black (D) backgrounds.

This study aimed to assess the effects of spatial and temporal environmental heterogeneity on the colour changing ability of *C. crangon*. Multiple behavioural lab experiments were conducted to analyse the influence of variation in illumination (over a day-night cycle), sediment colour and ease of burying on the background matching ability of this species, to gather a clearer understanding of the variability of colour adaptation in crustaceans. In order to test the effects of these factors, the following hypotheses were postulated: (i) background colouration and the presence of light both influence the colour of *C. crangon*; (ii) the brown shrimp is capable of fast repeated colour changes in response to temporal variability in background colour; (iii) background matching in the brown shrimp is mainly influenced by the colour of the sediment; (iv) shrimp inhibited from burying show enhanced colour changing abilities compared to shrimp that are able to bury; (v) long-term exposure to a black background reduces the brown shrimp's ability to adapt to a white background.

3.3. Methods

3.3.1. Collection and maintenance of the test organisms

Crangon crangon were collected by push net (1.2 mm^2 mesh size) in the Cleddau Ddu estuary (Lower Waterway) close to the town of Dale (Pembrokeshire, UK) on April 2015 and by bottom trawl (2 mm^2 mesh size) in Morecambe Bay (Flookburgh, UK) on June 2015. The first area is characterized by fine/muddy sand with gravel/rocks and green and brown algae (Carey et al., 2015), while in Morecambe bay the sediment at the sampling site (further away from the coast, reached at low tide) was characterized by more homogeneous brown mud. Shrimp were placed in aerated buckets and transported to the lab where they were acclimated in gently aerated 50L glass aquaria with a ~1 cm thick layer of black, white (Pettex Roman Gravel) or yellow (ProRep Desert sand SPD005) sand for at least a week prior to any experiments. Artificial sea water (Aquarium Systems, Instant Ocean) was used, with salinity and temperature (mean ± SD) maintained at 24.2 ± 5.9 PSU and 16.9 ± 2.3°C respectively. Caught *C. crangon* had a mean (±SD) total length (TL) of 50 ± 6 mm and a 5.5:1 Female:Male sex ratio. Prior to the experiments, to avoid cannibalism (Jung and Zauke, 2008, Hunter et al., 1998), shrimp were moved to individual 2L beakers (Ø: 13 cm) containing ~1 cm (0.1 dm^3) of sediment and 500 ml of artificial sea water, gently aerated. Beakers were wrapped in white or black paper (called “black” (BL) or “white” (WH) beakers) matching the sediment colour (unless stated otherwise), to avoid any external visual influence. Shrimp were fed *ad libitum* with fish, three times a week and any leftover food was removed two hours after feeding. Water was partially changed once per week. All shrimp were kept under a 12h:12h artificial illumination regime (Sylvania T5 830 fluorescent tube; light on: 8:00-20:00) but natural light from windows was not blocked (influencing the light cycle to a minor extent).

3.3.2. Colour measurements

Colour measurements were conducted following the protocol of chapter 2. Briefly, shrimp colouration was quantified as the percentage area covered by dark (black/brown) pigments (Brown and Wulff, 1941, Koller, 1927) on a section of the shrimp’s exopod of the tail fan (Brown and Wulff, 1941). Images of the exact centre (1 mm^2) of the right tail fan were made on a white stage under a Leica S6D dissecting microscope (Illumination: two JANSJÖ led

spotlights: 88 lm, 3000 Kelvin) and saved with Leica Application Suite v4.3.0. Dark pigment cover (PiC) was calculated using the colour threshold function (default thresholding algorithm and minimum manual adaptations; Chapter 2; Landini, 2015, Ridler and Calvard, 1978) in ImageJ (version 1.48; Schneider et al., 2012).

3.3.3. Biorhythm

To test whether the colour of *C. crangon* was subjected to a biorhythm, PiC was measured every three hours (Darnell, 2012) over the course of a full day-night cycle (starting at 09:00). Shrimp were randomly divided over black and white beakers and two different illumination treatments ("natural" and "reversed"). In the former treatment, beakers were kept under natural illumination, complemented by artificial illumination from 08:00 to 20:00; in the latter treatment beakers were kept in the dark with artificial light from 20:00 to 08:00. Temperature was maintained at 19.4 ± 1.1°C during the day and 19.8 ± 0.6°C at night. The shrimp were exposed for 84 hours to their assigned illumination regime, under the same conditions. Exposure to light during dark-period measurements was minimal (one minute required to take a photo). A total of 80 shrimp were tested over two day-night cycles.

3.3.4. Background matching

A series of four experiments were conducted to test the effects of variation in sediment characteristics on *C. crangon*'s background matching ability (Figure 3.2). For all experiments, shrimp were first acclimated in either black or white beakers and the initial PiC was estimated (Chapter 2). Colour change was measured before and one hour after moving shrimp to the opposite sediment colour (Figure 3.2A). The effect of repeated background changes (Figure 3.2B) was investigated by switching shrimp (N = 40) four times between black and white sediments (one hour time interval). The relative effects of colour of sediment and beaker sides (representing the surroundings above the sediment level) was assessed by varying both the colour of the sediment and beaker sides (Figure 3.2C), with all shrimp exposed to all four treatments (on different days) to account for individual variation.

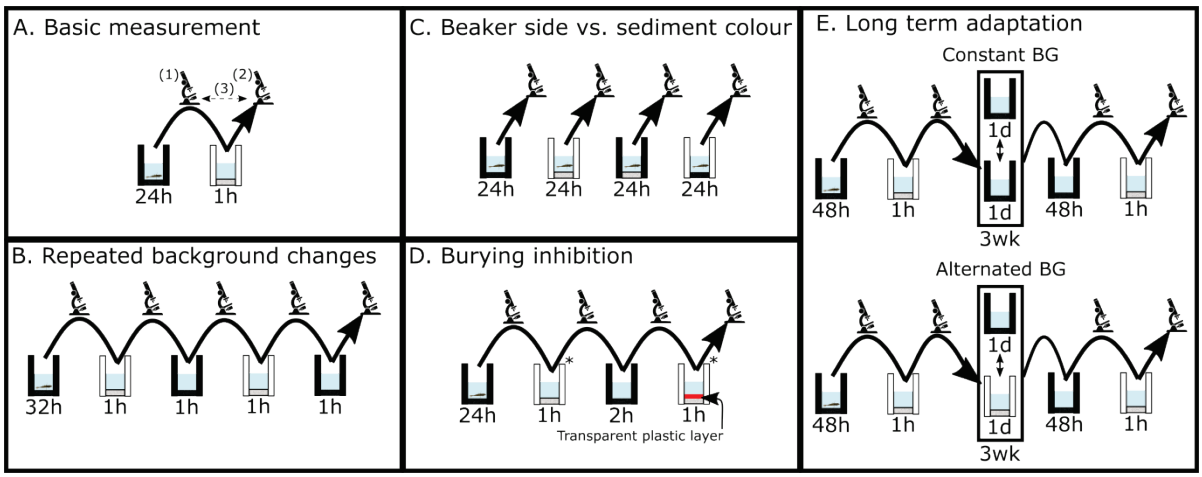

Figure 3.2. Schematic overview of the different protocols to determine the factors involved in *Crangon crangon* background matching. Arrows indicate shrimp being moved between beakers with different backgrounds, or to a dissecting microscope (microscope symbol) for pigment coverage measurements. Black and white colours indicate the colour of the sediment (bottom) and sides of the beaker. Time spent in each beaker is given below the beaker symbol. Repeated measurements with the same shrimp are indicated with continuous arrows. (1) First photo, (2) second photo, (3) colour change, * order randomly determined.

In all experiments, shrimp were allowed to bury, at least partially (Pinn and Ansell, 1993), in sand. To test the effect of burying inhibition (Figure 3.2D), shrimp (N = 32) were moved onto the opposite sediment colour for one hour and PiC was measured. These shrimp were then returned to the original coloured sediment for two hours before being placed again onto contrasting sediment for another hour. During the first or the last move, shrimp were placed in beakers with or without a plastic layer (burying prevented and control), in random order. Shrimp that did not bury when permitted were excluded from further analysis. In the repeated measurements and burying inhibition experiments (Figure 3.2B, D), each shrimp was tested starting from both colours, on different days.

Finally, long-term adaptation of *C. crangon* to a single or varying background colour(s) was tested keeping shrimp constantly on a black background (CST) compared with shrimp switched between backgrounds, alternating white and black backgrounds every other day (ALT; figure 3.2E). The PiC of shrimp (N = 15) before and one hour after movement from black to white sediment was assessed before and after three weeks of CST or ALT treatment. During this time, even CST shrimp were removed and replaced in their beakers to ensure equal levels of handling. To avoid any prior adaptation to black sediment, all shrimp were kept on yellow sediment for three weeks before the experiment started.

3.3.5. Data analyses

Nonparametric tests (Mann-Whitney U, Wilcoxon Signed Rank and Friedman's tests) were applied to test for differences in PiC and the degree of colour change between treatments (Barbiero, 2014, Zhao et al., 2001, Warton and Hui, 2010). The degree of colour change was estimated as the difference in PiC values (figure 3.2A), with positive values indicating that the animal became darker and negative values indicating that the animal became paler. Shrimp that died during the experiments were excluded from analysis. R statistical software v.3.1.2 (R Core Team, 2013) was used for data analysis.

Biorhythm was analysed using the glmmADMB R package (Bolker et al., 2012) to create a generalised linear mixed beta regression model (Cribari-Neto and Zeileis, 2010; Chapter 4). The fixed effects assessed were: sediment colour (WH, BL), presence of daylight (day: 05:00-21:00, night: 21:00-05:00), artificial illumination (on, off) and the time since the artificial light status changed (TLC). Shrimp ID was included as a random factor, in order to capture some of the autocorrelation in the model. A full model was constructed which contained interactions between all terms. Individual terms were removed from the model if their removal reduced the absolute value of the Akaike information criterion (AIC) by more than 2 (Burnham and Anderson, 2002).

3.4. Results

3.4.1. Biorhythm

Model selection based on lowest AIC resulted in a final model with sediment colour and daylight as independent effects and an interaction term between artificial illumination and TLC (Figure 3.3; Appendix 3.1). The model suggests that *C. crangon* are significantly (z = -6.07; P < 0.001) paler (lower PiC) on a white background than on a black background (independently of the presence of light or time of the day) and significantly (z = -5.38; P < 0.001) darker during the night than during the day (independently of the sediment colour and the presence of light). The interaction between artificial illumination and TLC can be interpreted as a significant (z = -3.18; P < 0.01) progressive darkening of *C. crangon* after the artificial light was turned off. Plots of individual shrimp showed large intra- and inter-individual variation in pigment cover over a day-night cycle (Appendix 3.2).

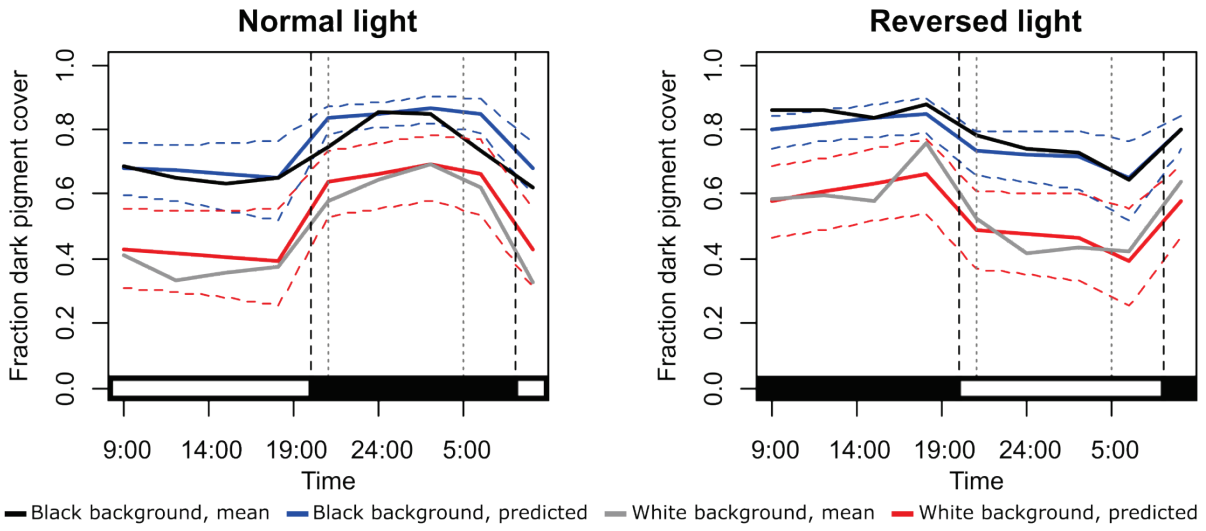

Figure 3.3. Effect of background colour and light on *Crangon crangon* mean dark pigment cover over a day-night cycle. The illumination regime is indicated with a black/white bar at the bottom of each graph. Solid lines: mean and predicted values on black and white backgrounds; dashed horizontal lines: confidence interval predictions; black dashed vertical lines: moment of light switch; period between grey dashed vertical lines: no day light. Measurements were made every 3 hours. N = 20 per treatment.

3.4.2. Repeated background changes

Crangon crangon were significantly darker (higher PiC) when acclimated for 24 hours in black beakers than when acclimated in white beakers (Time 0:00; Figure 3.4; Wilcoxon Test; N = 33, Z = -4.565, P < 0.001). Shrimp that were repeatedly transferred between black and white beakers showed a consistent significant higher PiC in black beakers compared to white

beakers, independent of the colour they were acclimated in (Figure 3.4; Friedman's Test with Bonferroni post-hoc correction: black acclimated: N = 33, X^2 = 43.32, P < 0.001; White acclimated: N = 31, X^2 = 58.10, P < 0.001). The specimens showed a consistent median change of 15% (mean: 20%) when moved between different backgrounds, independently of background colour (only the sign changed between positive and negative; Friedman's Test on absolute values: N = 31, X^2 = 4.085, P = 0.770). Plots of individual shrimp showed large intra- and inter-individual variation in pigment cover (Appendix 3.3) and a two-way ANOVA showed a significant influence of shrimp ID (Df = 30, F = 5.88, P < 0.001) and no influence of sediment colour (Df = 1, F = 0.351, P = 0.554) and acclimation colour (Df = 1, F = 0.786, P =0.376) on absolute colour change (%).

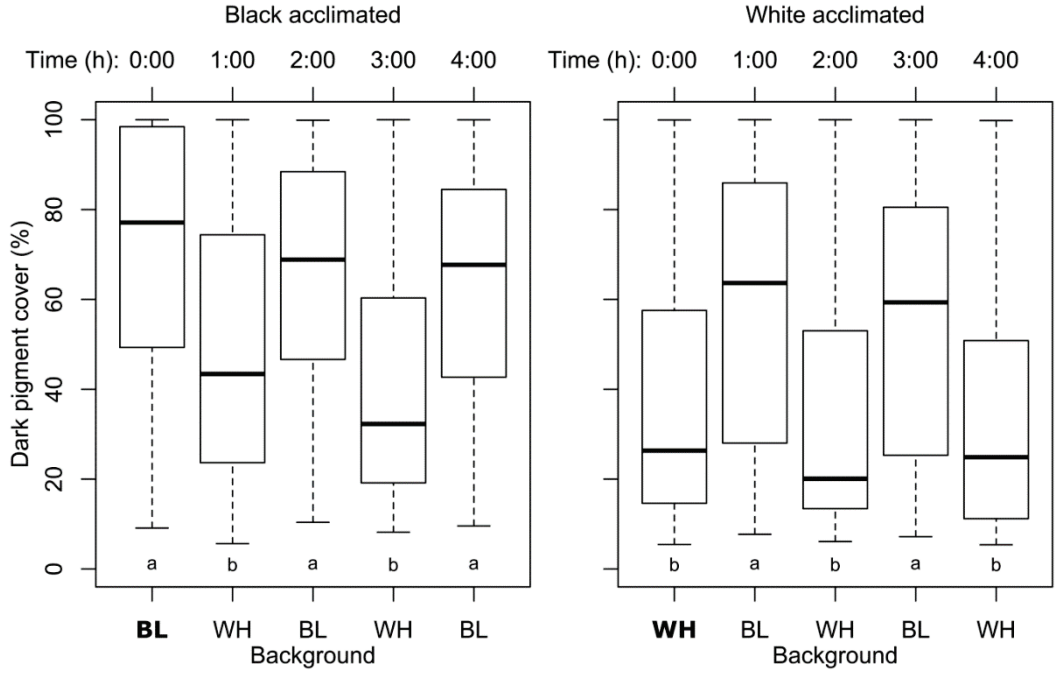

Figure 3.4. Box-and-whisker plots of dark pigment cover (%) during repeated shifts of *Crangon crangon* (N = 33) between black (BL) and white (WL) backgrounds. First measurement (in bold) was performed after 24h acclimation and all subsequent measurements after 1 hour permanence on the respective background. Time: number of hours after acclimation. Different letters indicate groups that are significantly different (P < 0.01) based on Dunn-Bonferroni corrected post-hoc analyses.

3.4.3. Sediment vs. beaker colour

Shrimp kept for a full day-night cycle in beakers with different combinations of sediment (BL or WH) and side colouration (BL or WH beaker) showed significant differences in PiC (Friedman's Test: N = 32, X^2 = 22.9, P < 0.001). Dunn-Bonferroni corrected pairwise comparisons showed that PiC varied significantly with sediment colour but not with beaker colour (Figure 3.5).

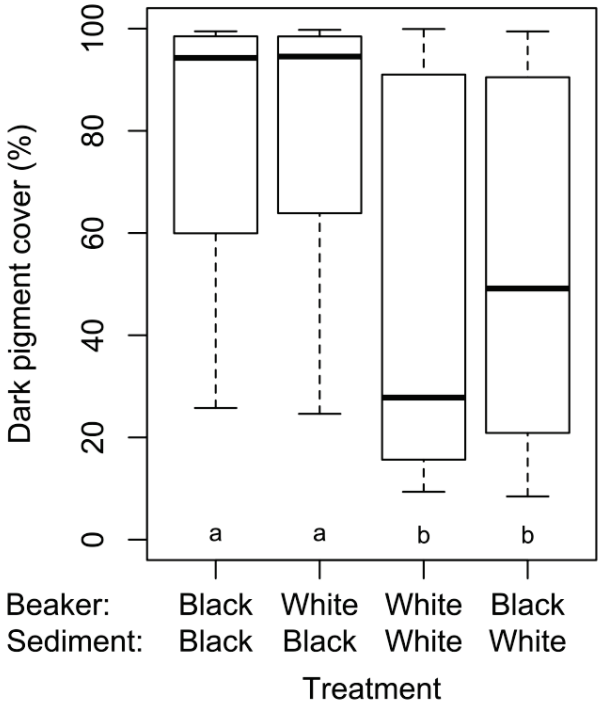

Figure 3.5. Box-and-whisker plots of dark pigment cover (%) of *Crangon crangon* (N = 32) kept for 24h in beakers with different combinations of sediment and beaker colours. Different letters indicate groups that are significantly different (P < 0.01) based on Dunn-Bonferroni corrected post-hoc analyses.

3.4.4. Burying inhibition

Preventing *C. crangon* from burying resulted in darker shrimp, regardless of the background colour. Shrimp that were transferred from black to white backgrounds did not change colour as much when their burying behaviour was inhibited compared to shrimp that could bury (Figure 3.6A; Wilcoxon Test; N = 26, Z = -2.248, P < 0.025) resulting in no median difference in PiC before and after the transfer (Appendix 3.4; median PiC BL = 92%; median PiC WH = 96%; Wilcoxon Test; N = 25, Z = -0.748 P = 0.455). On the contrary, *C. crangon* transferred from white to black backgrounds showed a significant larger median change in colour when their burying behaviour was inhibited than when they could bury (Figure 3.6B; Wilcoxon Signed Ranks Test; N = 22, Z = -3.750, P < 0.001). A total of 10 black acclimated and 8 white acclimated specimens were excluded from analysis because they did not bury when allowed. The acclimation time (24 hours vs. 2 hours; Figure 3.2D) did not influence *C. crangon* PiC values during the initial measurements (Wilcoxon Test: WH: N = 32, Z = -1.215, P = 0.224; BL: N = 31, Z = -1.568, P = 0.117).

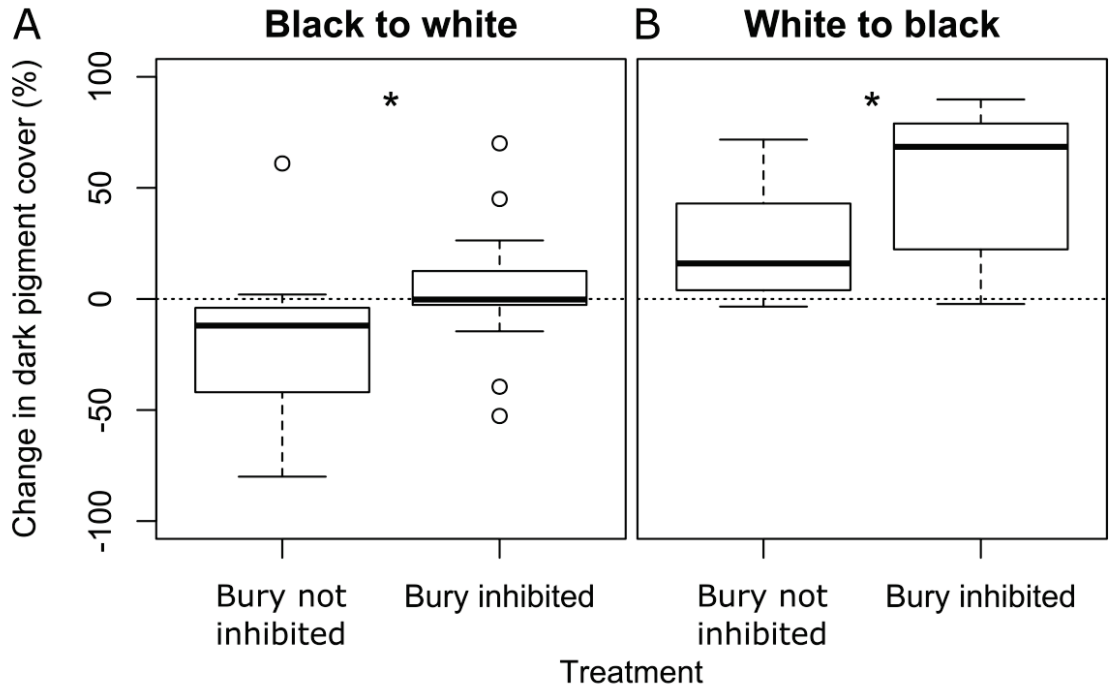

Figure 3.6. Effect of burying inhibition on *Crangon crangon* colour change. Box-and-whisker plots show the change in dark pigment cover within one hour of shrimp transferred to the opposite coloured background. A) Shrimp were tested after acclimation on a black background and after being kept for one hour on a white background (N = 25). B) Shrimp were tested after acclimation on a white background and after being kept for one hour on a black background (N = 22). The dashed line represents the level of no change. *: $P < 0.05$. Change in pigment cover was calculated from PiC values shown in Appendix 3.4.

3.4.5. Long term adaptation

Long term exposure to a single background colour had a negative impact on the ability of *C. crangon* to change colour. When transferred from black to white backgrounds, shrimp that were exposed exclusively to black backgrounds for 21 days showed a decreased ability to match the white background compared to their initial ability, as measured at the beginning of the experiment (day 0; Figure 3.7A; Wilcoxon Test: N = 11, Z = -2.934, $P < 0.01$; Appendix 3.5; median PiC BL = 65%; median PiC WH = 89%; Wilcoxon Test: N = 11, Z = -1.778, P = 0.075). On the contrary, shrimp that were exposed to daily switches between backgrounds maintained their ability to match the colour of the background (Appendix 3.5; median PiC BL = 65%; median PiC WH = 37%; Wilcoxon Test: N = 15, Z = -2.613, $P < 0.01$) and showed no difference in colour change between the initial and final measurements (Figure 3.7B; Wilcoxon Test: N = 15, Z = -0.227, P = 0.820). A total of four shrimp died during this experiment and were excluded from analysis.

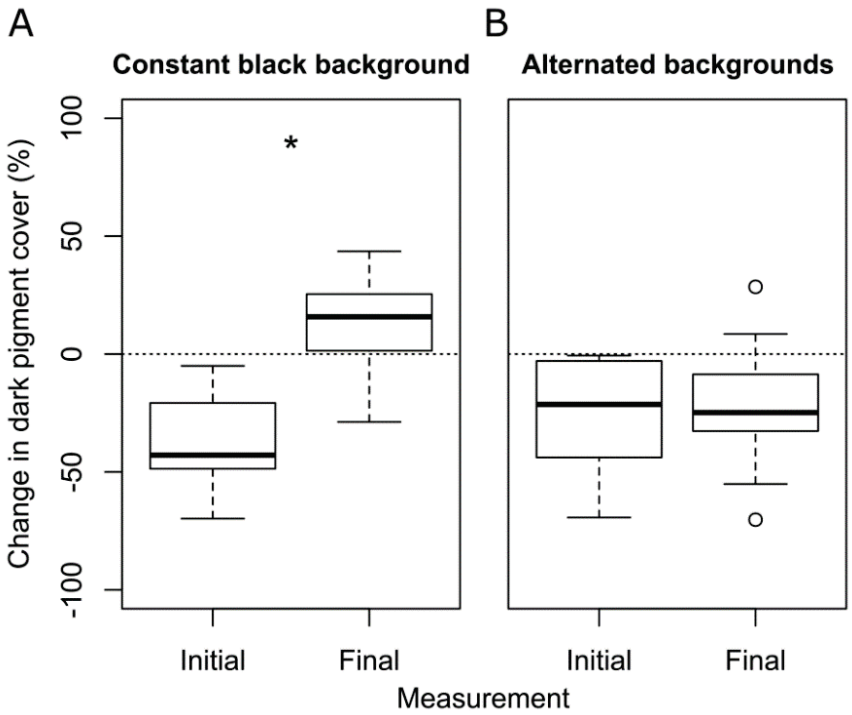

Figure 3.7. Effect of long term background adaptation on *Crangon crangon* colour changes. Box-and-whisker plots show the change in dark pigment cover within one hour of shrimp transferred from a black to a white background. Shrimp were first tested at day 0 (initial), than exposed to either (A) a constant black background (N = 11) or (B) daily alternating black and white backgrounds (N = 15), and tested a second time at day 21 (final). *: $P < 0.05$.

3.5. Discussion

Background adaptation of crustaceans is a complex process, influenced by multiple parameters (Umbers et al., 2014). These can be intrinsic, such as sex (Chassard-Bouchaud, 1965, Bauer, 1981), moult stage (Chassard-Bouchaud, 1965), stress (Detto et al., 2008), and extrinsic such as spatial and temporal environmental factors, including temperature (Brown and Sandeen, 1948, Smith, 1930, Stevens et al., 2013), UV radiation (Darnell, 2012), circadian rhythm (Darnell, 2012), tides and predation (Palma and Steneck, 2001, Manríquez et al., 2008). Here, I have focused on variation in illumination and sediment colour on the colour change abilities of *C. crangon*.

The overall colour of *C. crangon* showed a clear rhythm, being darker during the night and paler during the day. Other crustaceans also show similar colour patterns regulated by a circadian rhythm, as the horned ghost crab *Ocypode ceratophthalmus* (Stevens et al., 2013), even though in the majority of the species the pattern is inversed (darker during the day than at night; Bauer, 2004), as in the fiddler crab *Uca panacea* (Darnell, 2012). This nocturnal colouration was already observed at the end of the 1800 century in *Hippolyte varians* (Keeble and Gamble, 1899). Darker colouration during the day might reflect the use of chromatophore expansion as protection to UV light rather than a strategy to match the background. Indeed, also in *C. crangon*, there is a primary response (Chassard-Bouchaud, 1965) to light (with expansion of black pigments in the chromatophores) in response to increased intensity of incident light. In this experiment though, light intensity was not changed. Only absence-presence of light (mimicking night-day variations) was tested. These rhythms observed between absence-presence of light might facilitate either concealment/camouflage, energy saving, thermoregulation, UV protection, or a combination of factors, which might change with life history stages and sex (Duarte et al., 2017, Stevens et al., 2013, Powell, 1962, Kronstadt et al., 2013, Fingerman and Tinkle, 1956). In this study, I recorded a darkening of the colour during the night independently of the illumination provided to the shrimp, but I cannot definitely call this a circadian rhythm since no experiments were conducted in constant darkness (as would be required to check the endogenous process linked to colour change; Brown, 1950). Background matching during the full day-night cycle may enable *C. crangon* to camouflage itself during low light conditions, when shrimp are more active (Pihl and Rosenberg, 1984, Tiews, 1970, Al-Adhub and Naylor, 1975) and possibly more prone to predation. Thermoregulation and UV protection are, on

the other hand, likely of secondary importance in this case, since adult *C. crangon* live for the majority of the time submerged in temperate and turbid estuaries, almost completely buried in the sediment (Campos and van der Veer, 2008, Tiews, 1970, Siegenthaler et al., 2015, Belzile et al., 2002, Stevens et al., 2013). They might be more important at the pelagic larval stage (Figure 3.1B), as reported in other decapods (Anger, 2001, Miner et al., 2000).

Background matching is an important camouflage strategy of *C. crangon*, as already described since the early 1900s (Koller, 1927). Changes in background colouration resulted, on average, in a 20% change in dark pigment cover within one hour, this change being constant over repeated background switches. A constant colour change rate is in contrast to the behaviour of several species of flatfish, where melanophore response rates increase during repeated background switches (Burton and Driscoll, 1992, Osborn, 1939). Variation between individual *C. crangon* was high, which was compensated by repeated measurements on the same animal. Comparable rates of changes are observed in several other species of decapods (Umbers et al., 2014), but some crustaceans show, in contrast to *C. crangon*, differences in the rate of change between pigment dispersion and concentration (Llandres et al., 2013, Brown, 1950). C*rangon handi* is, for example, more successful in adapting to dark- than light-coloured substrates (Kuris and Carlton, 1977), while the opposite has been recorded in the ghost crab *O. ceratophthalmus* (Stevens et al., 2013). The costs of pigment dispersion and concentration are, however, not fully understood (Fuhrmann et al., 2011, Auerswald et al., 2008, Miner et al., 2000, Umbers et al., 2014, Duarte et al., 2017).

Sediment colour is the main factor determining *C. crangon* colouration and in the field shrimp with naturally variable occurring colours have been observed (Figure 3.8). Colours of structures above the sediment level (mimicked by the colour of the side of experimental beakers) did not have an effect on the shrimp's colour. The ability to match the colour of the sediment provides a camouflage advantage when shrimp emerge on light sediment besides darker structures, e.g. rocks. Background adaptation depends on the ratio of the light reflected from the environment to incident light (Burton, 2010, Brown and Sandeen, 1948, Pautsch, 1953, Fingerman and Lowe, 1957). Due to its low profile, the majority of the light conveyed in *C. crangon*'s eyes will be reflected from the sediment, explaining the low relevance of vision of other nearby objects for background matching. This also explains the good matching colour of buried individuals (as the eyes receive the stimulus even when the

body of the shrimp is covered by the substrate). Keeping its eyes above the sediment (Pinn and Ansell, 1993) allows the shrimp to continuously respond to light stimuli while buried, avoiding conspicuousness when emerging from the sediment (Stevens et al., 2013).

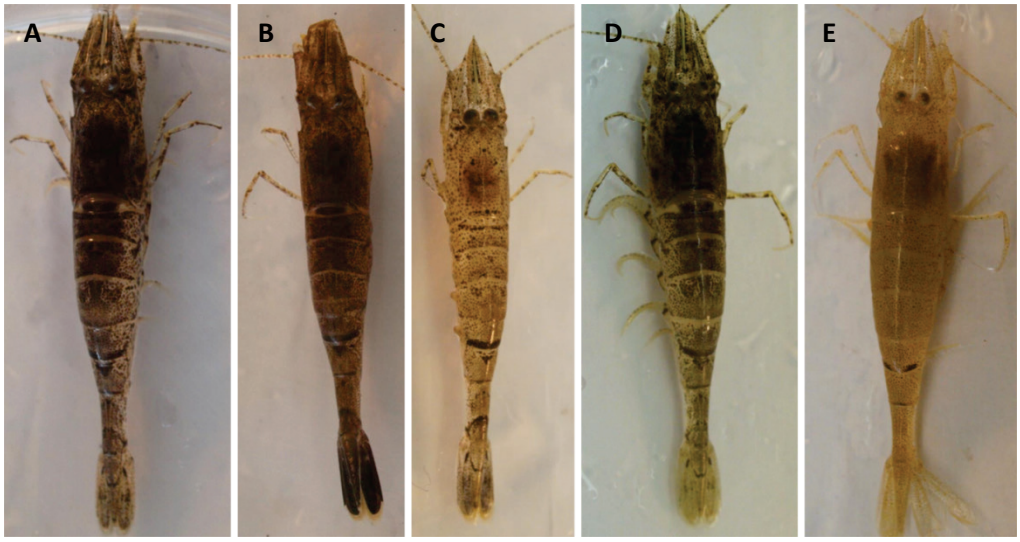

Figure 3.8. Examples of *Crangon crangon*'s variation in natural colouration. Specimens were caught in the beach in front of Dale, Pembrokeshire, in the Cleddau Ddu estuary. A-C: shrimp collected from muddy-sandy substrates; D: shrimp collected from algae-dominated sand; E: shrimp hold in a yellow bucket (notice the high spread of yellow pigments in chromatophores).

Patchiness in sediment colouration and ease of burying (e.g. due to sediment compactness) might also influence *C. crangon* colour. Long-time exposure to constant dark sediments resulted in darker shrimps during colour-change experiments. Background matching mechanisms likely vary among time and spatial scales (Stevens et al., 2014). Individuals inhabiting environments with more heterogeneous/patchy colours might be better adapted to reply to background changes than individuals that are exposed to a single background for a prolonged period of time (Fingerman and Lowe, 1957, Burton, 2010). Chromatic adaptations to a single background colour could further be influenced by the behaviour of the species, being more pronounced in species inhabiting in more consistent environment compared to others, such as *Carcinus maenas* (Powell, 1962), exposed to multiple habitats due to active daily movements. Indeed, active responses to visual predators in crabs (habitat choice when predatory clues are detected) (Manríquez et al., 2008) can be combined with colour morphs, especially in juveniles (Palma and Steneck, 2001). In the brown shrimp, juveniles do not present variable colour morphs, as the overall cryptic strategy is to burrow and match the substrate. Inhibition in *C. crangon* burying behaviour also resulted in darker shrimps, independently of the sediment colour. Darkening of the shrimp might in this case

represent a "visual stress sign" to unfavourable environmental conditions. Studies on multiple species of fish have shown a link between pigment regulation and stress responses for melanin dispersing hormones (Balm et al., 1995, Van der Salm et al., 2004, Lamers et al., 1992) and several invertebrate species are known to become darker or red when subjected to handling stress (Detto et al., 2008, Auerswald et al., 2008, Feyjoo et al., 2011, Perazzolo et al., 2002), including the fiddler crab *Uca vomeris* (Hemmi et al., 2006). This possible link between environmental stressors and background adaptations represents a very interesting topic for further research, as behavioural responses can be early sign of stressors in animals. Even in lab experiments, responses to stressors should be taken into account, especially considering that essential works on *C. crangon* colour changes have been performed in aquaria without a sediment layer (Koller, 1927, Chassard-Bouchaud, 1965), which is known to be a major stressor to the shrimp (Paschke et al., 2004, Hagerman, 1970).

Habitat characteristics such as light, sediment colour and sediment compactness vary across spatial and temporal scales in heterogeneous and dynamic habitats as estuaries and can influence *C. crangon* colouration and background matching (Figure 3.8). Our results confirm that this behaviour depends on an interlinked set of environmental and behavioural parameters (Endler, 1995, Stevens et al., 2014). The complexity of this behavioural response results in high inter- and intra-individual variation in background matching and mismatching, as has been observed in *Crangon* spp. (Brown and Wulff, 1941, Koller, 1927) and other crustaceans (Stevens et al., 2014, Stevens et al., 2013, Duarte et al., 2017). Phenotypic variations, resulting in well distinguished morphotypes are present in many crustaceans (e.g., *H. obliquimanus*; Duarte et al., 2016) and could be produced by genetic polymorphism or plasticity. Such variability in colour is not restricted to crustaceans and can play a role in speciation in a variety of taxa (McLean and Stuart-Fox, 2014). In *C. crangon* specific colour morphs are not present, not even in the juvenile stages, as it occurs in other crustaceans (Palma and Steneck, 2001), rather a variety of responses to match the substrate across individuals as well as in the same individual when recorded for prolonged periods of time. These variations might combine a mix of adaptive and non-adaptive (including stress) responses (Alonzo, 2015) and/or can make the overall populations more adapt to sudden changes (especially in a world where human impacts are sudden and unpredictable). Identifying individual variation and the mechanisms behind this variation is essential for assessing ecological and evolutionary processes (Bolnick et al., 2003). Besides identifying

possible drivers for inter- and intra-specific variation, studies on colour change also provide information on how animals adapt to different environments and on the relationship between visual perception and animal colouration (Stevens et al., 2013). The inter- and intra-specific variation recorded under controlled lab conditions is expected to be even amplified in the field. In those conditions, the main key ecological drivers influencing colour change will be acting concurrently. Once mechanisms and specific adaptations are uncovered and interpreted, lab experiments should be paralleled by field experiments. This study demonstrates that the ability of animals to change colour is a delicate balance of behavioural-plasticity and environmental adaptation at different spatial/temporal scales (Todd et al., 2012). Failure to adapt to the right colour under the right circumstance influences survival (Miner et al., 2000, Magige et al., 2008, Mueller and Neuhauss, 2014) so the prioritization of the factors influencing background matching should be under strong selection pressure. Taking into account the factors that play a role in determining animal colouration is, therefore, an essential step in the understanding of the evolution of animal colour change in heterogeneous and dynamic habitats.

Chapter 4.

DNA metabarcoding unveils multi-scale trophic variation in a widespread coastal opportunist

4.1. Abstract

A thorough understanding of ecological networks relies on comprehensive information on trophic relationships among species. Since unpicking the diet of many organisms is unattainable using traditional morphology-based approaches, the application of high-throughput sequencing methods represent a rapid, powerful and reliable way forward in this field. Here, I assessed the application of DNA-metabarcoding with nearly universal primers for the mitochondrial marker cytochrome c oxidase (COI) in defining the trophic ecology of adult brown shrimp, *Crangon crangon*, in six European estuaries. The exact trophic role of this abundant and widespread coastal benthic species is somewhat controversial, while information on geographical variation remains scant. Results revealed a highly opportunistic trophic behaviour, with 2429 molecular operational taxonomic units (306 of which were identified to the species level), belonging to 35 phyla, detected in its diet. Predominant species included other abundant coastal and estuarine taxa, such as the shore crab *Carcinus maenas* and the amphipod *Corophium volutator*. Jacobs' selectivity index estimates based on DNA extracted from both shrimp stomachs and sediment samples indicated a high preference for arthropods, annelids and fish. Spatial variation in diet composition, both at regional and local scales, confirmed the highly flexible nature of this trophic opportunist. Furthermore, the detection of a prevalent, possibly endoparasitic fungus (*Purpureocillium lilacinum*) in the shrimp's stomach demonstrates the wide range of questions that can be addressed using metabarcoding, towards a more robust reconstruction of ecological networks.

4.2. Introduction

Trophic interactions provide important insights on a wide range of ecological dynamics, ranging from individual to ecosystem levels and including animal behaviour, predator-prey interactions, food web structure and community ecology (Pinol et al., 2014, Leray et al., 2015, Van Tomme et al., 2014). The feeding strategy of key consumers can have pronounced influences on ecosystem dynamics (Hanski et al., 1991, Holling, 1965) and their stomach contents can reveal essential information on food item distribution and prey assemblage structure (Lasley-Rasher et al., 2015). Crustaceans are a key component in marine/estuarine soft bottom habitats (Navia et al., 2016, Evans, 1983, Evans, 1984) and evaluating their diet is very challenging due to the complexity of direct observations on predation rates and the limitations associated with the identification of partially digested food items (Feller, 2006, Asahida et al., 1997, Symondson, 2002).

The recent application of high-throughput sequencing (HTS) tools, such as metabarcoding, promises to revolutionise the way prey diversity and composition are estimated from gut contents or faeces of consumers (Kartzinel and Pringle, 2015, Leray et al., 2015). Metabarcoding refers to the identification of multiple species (or other taxonomic ranks) based on bulk DNA extracted from community (many individuals and multiple species) or environmental samples (i.e. water, soil, faeces; Barnes and Turner, 2016), by means of massive parallel sequencing of polymerase chain reaction (PCR) amplicons generated using primers of varying universality (Barnes and Turner, 2016, Taberlet et al., 2012a, Leray et al., 2015, Pompanon et al., 2012, Kartzinel and Pringle, 2015). Metabarcoding has proved to be highly effective for the identification of prey remains with improved taxon resolution, accuracy and speed of analysis, compared to traditional morphological methods (Berry et al., 2015, Casper et al., 2007, Symondson, 2002). Yet, some challenges remain, such as fragmentation of partially-digested DNA, variability in taxon-specific digestion rates, secondary predation, and, typically, the presence of high proportion of DNA from the study organisms itself, which may reduce sequencing depth and render cannibalism undetectable (Berry et al., 2015, Pinol et al., 2014, Barnes and Turner, 2016).

The brown shrimp, *Crangon crangon* (L.) is a key crustacean species in European coastal waters. Its wide distribution from the White Sea to Morocco, year round occurrence and high abundance make it an essential part of the benthic food web (Bamber and Henderson, 1994, Campos and van der Veer, 2008, Ansell et al., 1999, Hostens and Mees, 1999, Evans,

1984). *Crangon crangon*'s diet includes a wide variety of prey species, from meiofauna to fish (Oh et al., 2001, Tiews, 1970, Evans, 1983), including juvenile stages of several commercially important teleosts and bivalves (van der Veer and Bergman, 1987, van der Veer et al., 1998). As a juvenile, it relies mostly on the consumption of meiofaunal prey items while it switches to larger demersal organisms as an adult, including conspecifics (cannibalism occurs in larger shrimp)(Oh et al., 2001, Pihl and Rosenberg, 1984, Evans, 1984). The trophic position of *C. crangon* is still under discussion, being described as a trophic generalist (Evans, 1983), carnivorous opportunist (Pihl and Rosenberg, 1984) omnivorous (Raffaelli et al., 1989, Tiews, 1970, Lloyd and Yonge, 1947, Ansell et al., 1999) and probable scavenger (Ansell et al., 1999). To some extent, such uncertainty can be caused by a flexible and broad trophic niche breadth but also by the fact many studies have relied on microscopic identification of prey remains (e.g. Boddeke et al., 1986, Oh et al., 2001) but see also (Nordström et al., 2009). Yet, prey items are usually macerated to a fine degree by *C. crangon*, and a high proportion of its stomach contents is, consequentially, hardly identifiable by morphological examination (Asahida et al., 1997, Wilcox and Jeffries, 1974). Quality and reliability of results are therefore affected by the subjectivity and taxonomic expertise of the observers. Furthermore, most studies to date have focused on a limited number of locations and relatively small spatial scales and narrow size ranges (e.g. Pihl and Rosenberg, 1984, Evans, 1984, Oh et al., 2001). Environmental variables, such as temperature, salinity and sediment characteristics, can have pronounced impacts on benthic communities and show large spatial and temporal variation in estuarine systems (Sousa et al., 2007). The trophic ecology of *C. crangon* is expected to vary reflecting changes in environmental conditions and prey availability across European coasts (Oh et al., 2001, Pihl and Rosenberg, 1984, Pihl, 1985).

Here, I report on a large-scale analysis of the trophic ecology of *C. crangon,* which reveals its ecological role in estuarine systems. By using nearly universal primers for mitochondrial cytochrome c oxidase I, I assessed the application of metabarcoding to describe the diet of this crustacean and aimed to reveal variation in its trophic ecological function on a European scale. More specifically, I tested whether metabarcoding can (a) provide a detailed and objective overview of *C. crangon* diet, including prey selectivity, using DNA extracted from stomach and environmental samples; (b) identify geographical patterns in its trophic

ecology, both at local and regional scales and (c) assess consistent and general trophic patterns in order to better define the ecological role of this ubiquitous species.

4.3. Methods

4.3.1. Sample collection and processing

Brown shrimp and sediment samples were collected from 24 sites distributed over 6 estuaries in the Netherlands, Portugal and the United Kingdom (Figure 4.1). Adult shrimp (>20 mm total length, TL; tip of the rostrum to tip of the telson) were captured in the intertidal zone (0-1m depth) by push-net at low tide (±3h). Shrimp (30-50 per site) were placed on ice and transported to the lab to be stored at -20 °C. Sediment was collected for the extraction of environmental DNA to characterise the biological community present at each site. Sediment was sampled from the upper 2 cm surface layer, which represent the most recent DNA deposits (Turner et al., 2015, Limburg and Weider, 2002), with a PVC corer (3.2 mm Ø). Per site, 3 sediment subsamples were collected at several meters distance from each other and combined to reduce the influence of local heterogeneity (Taberlet et al., 2012b). The sediment was stored in 96% ethanol, transported on ice and kept at -20 °C. At each site, temperature, salinity (Fisher Scientific Traceable Salinity Meter), pH (Hanna HI 98129), dissolved oxygen (OxyGuard Handy Mk I) and turbidity (Eutech TN-100) were measured in triplicates. Extra sediment was collected, in triplicates, from each site for granulometric analyses (Horiba LA-950 Particle size analyser) and Total Organic Matter (TOM) determination by means of ashing (550°C, 6h).

4.3.2. DNA extraction

Overall, 1025 shrimp (20-50 mm TL) were caught and 494 full stomachs (visual determination) were dissected using flame-sterilised tools to avoid cross contamination. To avoid contamination with eDNA originating outside of the shrimp, extra care was taken to avoid that stomachs came in contact with any external shrimp tissue. Stomachs were pooled in batches of 8 prior to DNA extraction (Deagle et al., 2005, Ray et al., 2016), resulting in maximum 3 samples per site. Due to a high percentage of empty stomachs in natural populations (20-60 %; Feller, 2006, Pihl and Rosenberg, 1984, Oh et al., 2001), some sites contained only 2 replicates and some replicates contained less than 8 full stomachs (see appendix 4.1): the latter, were still included in the analyses as variation in number of stomachs pooled did not affect the patterns observed (see results).

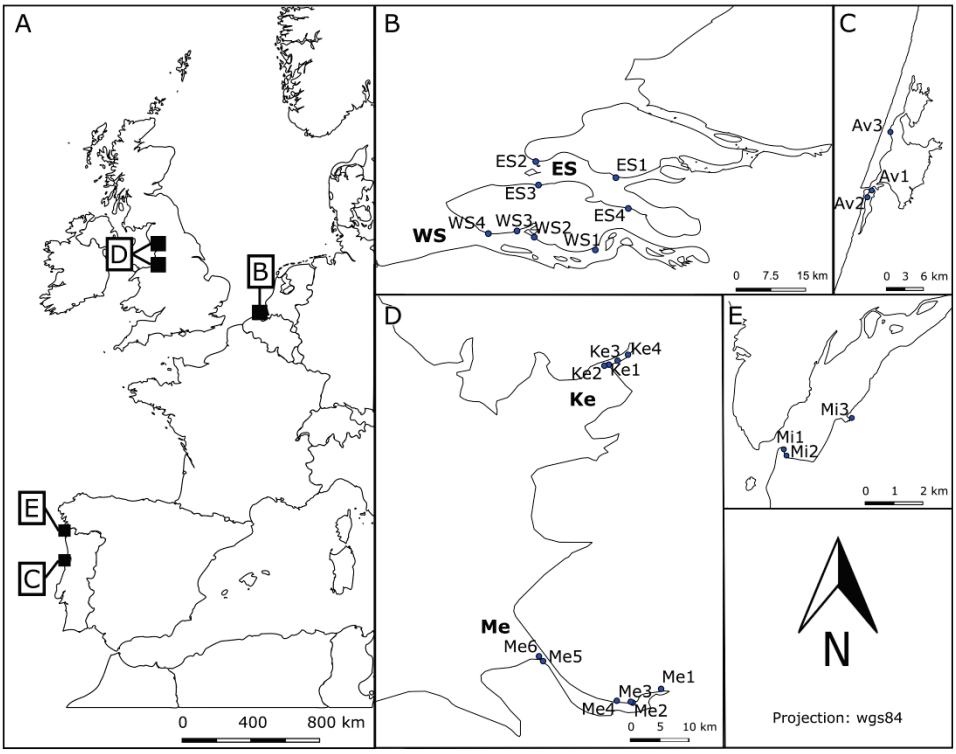

Figure 4.1. Overview of sample locations, illustrating (A) the overall western European scale; (B) the Dutch estuaries, Western Scheldt (WS) and Eastern Scheldt (ES); (C) the British estuaries, Mersey (Me) and Kent (Ke); the Aveiro (D) and Minho (E) estuaries in Portugal. Small dots within estuaries represent individual collection points for shrimp and sediment samples.
Source map: OpenStreetMap.

In addition to the full stomach samples, three pooled samples of 8 visually empty stomachs were included for comparative purposes. DNA was extracted from 0.25 g of homogenized pooled stomach contents using the PowerSoil [illegible] Kit (Mo-Bio laboratories), whereas DNA from sediment (10 g) was extracted using the PowerMax® DNA Soil Kit (Mo-Bio laboratories). A Qubit fluorometer (Thermo Fisher Scientific) was used to assess DNA concentrations of purified extracts. DNA extraction and pre-PCR preparations were performed in separate labs from post-PCR procedures to avoid contaminations.

4.3.3. DNA amplification and high-throughput sequencing

Amplification of DNA, for both stomach and sediment samples (including 2 extraction blanks), was achieved using a single set of versatile, highly degenerated PCR primers targeting the 313-bp Leray fragment (Leray et al., 2013) of the mitochondrial cytochrome c. oxidase subunit I (COI) region. The mICOIintF-XT primer (5'-GGWACWRGWTGRACWITITAYCCYCC-3') was used as forward primer. This modified version

(by Wangensteen et al., in review) of the mlCOIintF primer (Leray et al., 2013) included two extra wobble bases (equimolar mixtures of two different bases at a given position) and two inosine nucleotides (that can match any nucleotide) to enhance its eukaryotic universality. The reverse primer was jgHCO2198 (5'-TAIACYTCIGGRTGICCRAARAAYCA-3'; Geller et al., 2013). The Leray fragment has already been successfully applied for both the characterisation of marine communities and marine fish gut contents (Leray et al., 2013, Leray and Knowlton, 2015, Leray et al., 2015). Eight-base oligo-tags (Coissac et al., 2012) attached to the metabarcoding primers were added to the amplicons during a single PCR step, in order to label different samples in a multiplexed library (the same index sequence was applied to both the forward and reverse primer sets; Schnell et al., 2015a); moreover a variable number (2, 3 or 4) of fully degenerate positions (Ns) was added at the beginning of each primer, in order to increase variability of the amplicon sequences and thus improving the identification of clusters on the Illumina MiSeq flowcell during the initial sequencing cycles (Guardiola et al., 2015, De Barba et al., 2014). The PCR mix recipe included 10 µl AmpliTaq gold 360Master mix (Applied Biosystems), 3.2 µg Bovine Serum Albumin (Thermo Scientific), 1 µl of each of the 5 µM forward and reverse tagged-primers, 5.84 µl H_2O and 2 µl extracted DNA template (~ 5 ng μl^{-1}). The PCR profile included an initial denaturing step of 95 °C for 10 min, 35 cycles of 94 °C for 1 min, 45 °C for 1 min and 72 °C for 1 min and a final extension step of 72 °C for 5 minutes. After quality check of all amplicons by electrophoresis, the tagged PCR products (including 2 negative controls) were pooled into two multiplexed sample pools (sediment and stomach) and purified using MinElute columns (Qiagen)(O'Donnell et al., 2016). Two Illumina libraries were subsequently built from these pools, using the NextFlex PCR-free library preparation kit (BIOO Scientific). This library preparation kit ligates index sequences to the amplicons to distinguish libraries and adds adapter sequences to bind the amplicons to the sequencer flow cell (O'Donnell et al., 2016). Libraries were quantified using the NEBNext qPCR quantification kit (New England Biolabs) and pooled in a 1:4 sediment:stomach molar concentration ratio (similar to the sediment:stomach sample ratio) along with 0.7% PhiX (v3, Illumina) serving as a positive sequencing quality control. The libraries with a final molarity of 8 pM were sequenced on an Illumina MiSeq platform using v2 chemistry (2x250 bp paired-ends).

Preliminary analyses of the sequencing data revealed a large number of reads belonging to one Molecular Taxonomic Unit (MOTU) in the fungal order Hypocreales (Ascomycota). For

further identification, the ITS fragment was amplified from five samples with a high number (>90% read abundance) of reads of this MOTU, with the primer combination ITS1f (5'-CTTGGTCATTTAGAGGAAGTAA-3'; Gardes and Bruns, 1993) and ITS4ASCO (5'-CGTTACTRRGGCAATCCCTGTTG-3'; Nikolcheva and Bärlocher, 2004), specific for Ascomycota. The PCR mix recipe was similar to the one used for the Leray fragment described above and the PCR profile included an initial denaturing step of 95 ºC for 5 min, 32 cycles of 95 ºC for 30 sec, 55 ºC for 30 sec and 72 °C for 1 min and a final extension step of 72 ºC for 10 minutes (Manter and Vivanco, 2007). After electrophoresis check, the amplicons of these five samples were cleaned and Sanger sequenced by Source Bioscience Sequencing UK.

4.3.4. Bioinformatic and data analyses

Bioinformatic analyses were performed using the OBITools metabarcoding software suite (Boyer et al., 2016). Read quality assessment was performed with FastQC and paired-end read alignment using illuminapairedend, retaining reads with an aligment quality score > 40. Demultiplexing and primer removal was achieved using ngsfilter. Sequences were only retained if the same index tag was found on both the forward and reverse read (Andruszkiewicz et al., 2017). Obigrep was applied to select all aligned reads with a length between 303-323 bp and free of ambiguous bases. Obiuniq was used to dereplicate the reads and the uchime-denovo algorithm (Edgar et al., 2011) (implemented in VSEARCH; Rognes et al., 2016) was used to remove chimeras (3.1% of the sequences were found to be chimeras). Amplicon clustering was performed using the SWARM step-by-step aggregation algorithm (Mahé et al., 2015, Mahé et al., 2014) with a d value (local cluster radius) of 13 which offers a conservative solution to the high variability of the COI gene (Wangensteen et al., in review). Singletons were removed after clustering. Taxonomic assignment of the representative sequences for each MOTU was performed using the ecotag algorithm (Boyer et al., 2016), using a local reference database (Wangensteen et al., in review) containing filtered COI sequences retrieved from the BOLD database (Ratnasingham and Hebert, 2007) and the EMBL repository (Kulikova et al., 2004). This algorithm uses a phylogenetic approach to assign sequences to the most reliable monophyletic unit, based on the density of the reference database (Guardiola et al., 2015). The data was refined by clustering MOTUs assigned to the same species, abundance renormalization (Wangensteen and Turon, 2016) and by removing bacterial reads and contaminations of human or terrestrial origin

(Guardiola et al., 2015). Any MOTU with less than 5 reads per sample was removed on a sample-by-sample basis to avoid false positives and low frequency noise (De Barba et al., 2014; Wangensteen et al., in review, Leray and Knowlton, 2017, Giguet-Covex et al., 2014). The use of a minimum copy threshold is a widely employed strategy to remove sequencing artefacts (Alberdi et al., 2017).

All statistical analyses were performed in R v3.1.3 (https://www.R-project.org/) with the vegan (v2.3-5) and BiodiversityR (v2.5-3) packages (Oksanen et al., 2016, Kindt and Coe, 2005). Only MOTUs showing abundances ≥ 0.5% in the full stomach samples were considered for non-metric multidimensional scaling (nMDS; with 75%-confidence ellipses; Malaney et al., 2015), canonical correspondence analysis (CCA) and PERMANOVA analyses (Albaina et al., 2016). The influence of environmental variables (mean temperature, salinity, pH, oxygen saturation, turbidity, median sediment grain size and TOM) on the full stomach contents were tested by means of CCA and PERMANOVA. PERMANOVAs were calculated using the function Adonis (vegan) with Bray-Curtis dissimilarities and 1000 permutations. Prior to CCA and PERMANOVA, model selection was performed using the function ordistep (vegan). Prey MOTU richness was represented as MOTU accumulation curves after rarefaction for the number of reads (250 reads, 500 permutations) and the number of samples (9-15 samples, 1000 permutations). The Jacobs' Selectivity Index (Jacobs, 1974) was calculated based on the relative read abundances of the MOTUs (Kowalczyk et al., 2011) extracted from sediment and stomach samples in accordance to Jacobs (1974). Trophic significance of individual MOTU was determined based on the relative read abundance, fraction of samples with MOTU presence and Jacobs' Selectivity Index as follows: Trophic significance = relative abundance * fraction of samples * (Jacobs' Selectivity Index + 1). Trophic significance was represented in categorical terms based on the relative trophic significance of each MOTU (high: > 10 %, medium 1 % - 9 %, low < 1 %) instead of exact values since the relative abundances of individual taxa should be considered with caution (Deagle et al., 2005).

4.4. Results

4.4.1. Collection statistics

A total of 1025 *C. crangon* were caught with a 1:8 M:F sex ratio (based on 767 shrimp which could be sexed morphologically). About 7.5% of the females were ovigerous. Mean (±SD) wet weight was 0.40 ± 0.26 gram; mean (±SD) TL was 35.1 ± 7.6 mm and mean (±SD) carapace length (CL) was 7.4 ± 1.6 mm (CL = 0.214*TL; r^2 = 0.81, N =1025). TL varied significantly between sites (Appendix 4.2; One-way ANOVA: Df = 23, F = 47.95, P <0.001). Overall, the proportion of *C. crangon* with a full stomach was 57.9%. Mean proportion of full stomachs per site (58.9 ±19.3%) was not correlated with the time of sampling (Pearson's correlation: R^2 = 0.07, P = 0.754, N=24).

4.4.2. High-throughput DNA sequencing

A total number of 8,895,448 reads were obtained from an Illumina MiSeq run of pooled amplicon libraries built from 24 sediment samples, 69 pooled *C. crangon* full stomach samples (from now on referred to as stomach samples), three pooled *C. crangon* empty stomach samples (comprising of stomach tissue and clear liquid) and two PCR blanks. Variation in the number of pooled stomachs did not affect the patterns of diet composition (PERMANOVA: pseudo-F: 0.39, P < 0.6380) or MOTU richness (rarefied to 135 reads) per sample (generalized linear model with quasipoisson distribution: Z = 0.19, P = 0.456). In total, 5,704,471 reads remained after sample demultiplexing, quality and sequence-length filtering, and removal of bacterial reads, contaminations and false-positives (sediment samples: 742,286; stomach samples: 4,828,136; empty stomach samples: 134,049). Taxonomic assignment resulted in a total of 8,352 MOTUs, of which 6,447 MOTUs belonging to 40 phyla were detected in the sediment samples, 2,429 (35 phyla) in the stomach samples, and 17 (10 phyla) in the empty stomach samples. A total of 520 MOTUs were detected both in the sediment and stomach samples and only two (unassigned Rhodophyta and unassigned Eukaryota) were detected exclusively in the empty stomach samples. Of the total number of MOTUs detected, 595 could be assigned to the species level of which 306 were detected in the stomach samples. Mean (±SD) proportion of *C. crangon* reads was 28 ± 29% (range: 0.2-97.5%) in the stomach samples and 47 ± 46% (range: 10.6-99.0%) in the empty stomach samples. Mean proportion of *C. crangon* reads was 1 ± 4% in the sediment

samples (range: 0.0-21.0%). Remaining number of reads per sample ranged 179-203,808 in full stomach, 7-332 in empty stomach and 5,114-71,770 in sediment samples. A high number of reads (4,828,136 reads) belonging to a fungus of the species *Purpureocillium lilacinum* (Ascomicota: Hypocreales) was detected in almost all (95%) stomach samples and identified using both COI (100% identity) and ITS markers (100% identity; appendix 4.3). Mean (±SD) proportion of *P. lilacinum* reads was 36 ± 37 % (range: 0.0-97.4%) in full stomach, 53 ± 47 % (range: 0.1-89.3%) in empty stomach and 0.1 ± 0.2 % (range: 0.0-0.8%) in sediment samples. All *C. crangon* and *P. lilacinum* reads were removed from the database prior to further analyses on diet, resulting in a total of 2,691,998 reads. The final number of reads per stomach sample varied near-randomly, without systematic trends across estuaries (Appendix 4.4), indicating that diversity estimates were not affected by sequencing depth.

4.4.3. Description of *Crangon crangon* diet

Analysis of *C. crangon* stomach contents showed large variation in relative MOTU abundances between samples (Figure 4.2). Notable patterns are the lack of a dominant MOTU detected in stomachs from the Aveiro estuary; a relatively high contribution of the decapod crabs *Carcinus maenas* and *Pisidia longicornis* in the Minho estuary; the detection of the introduced barnacle *Austrominius modestus* in the Scheldt and Mersey estuaries; high amounts of the polychaete *Pista cristata* in the Eastern Scheldt; the substantial proportion of the mysid *Neomysis integer* reads in the Mersey estuary; the large contribution of the amphipod *Corophium volutator* in the Kent estuary. In general, the shore crab *C. maenas* and the amphipod *C. volutator* were the trophically preponderant prey items for *C. crangon* (Table 4.1). Other important MOTUs included annelids (*Hediste diversicolor* and *P. cristata*), other amphipods (*Bathyporeia sarsi*), other decapods (*P. longicornis*), chironomids (unassigned), mysids (*N. integer*), barnacles (*A. modestus*), molluscs (*Patella rustica*) and picoplankton (*Micromonas* sp.). Fish reads were detected in all estuaries with a total of 22 species present in 29 stomach samples. Five fish species were relatively abundant (≥ 5%; Table 4.1) but were generally only present in a low number of stomach samples. There was a high discrepancy between MOTU abundances in the stomach (Figure 4.2) and sediment samples (Figure 4.3), resulting in many MOTUs having a maximum Jacobs' selectivity index value of 1 which indicates the prey items being highly selected (Table 4.1).

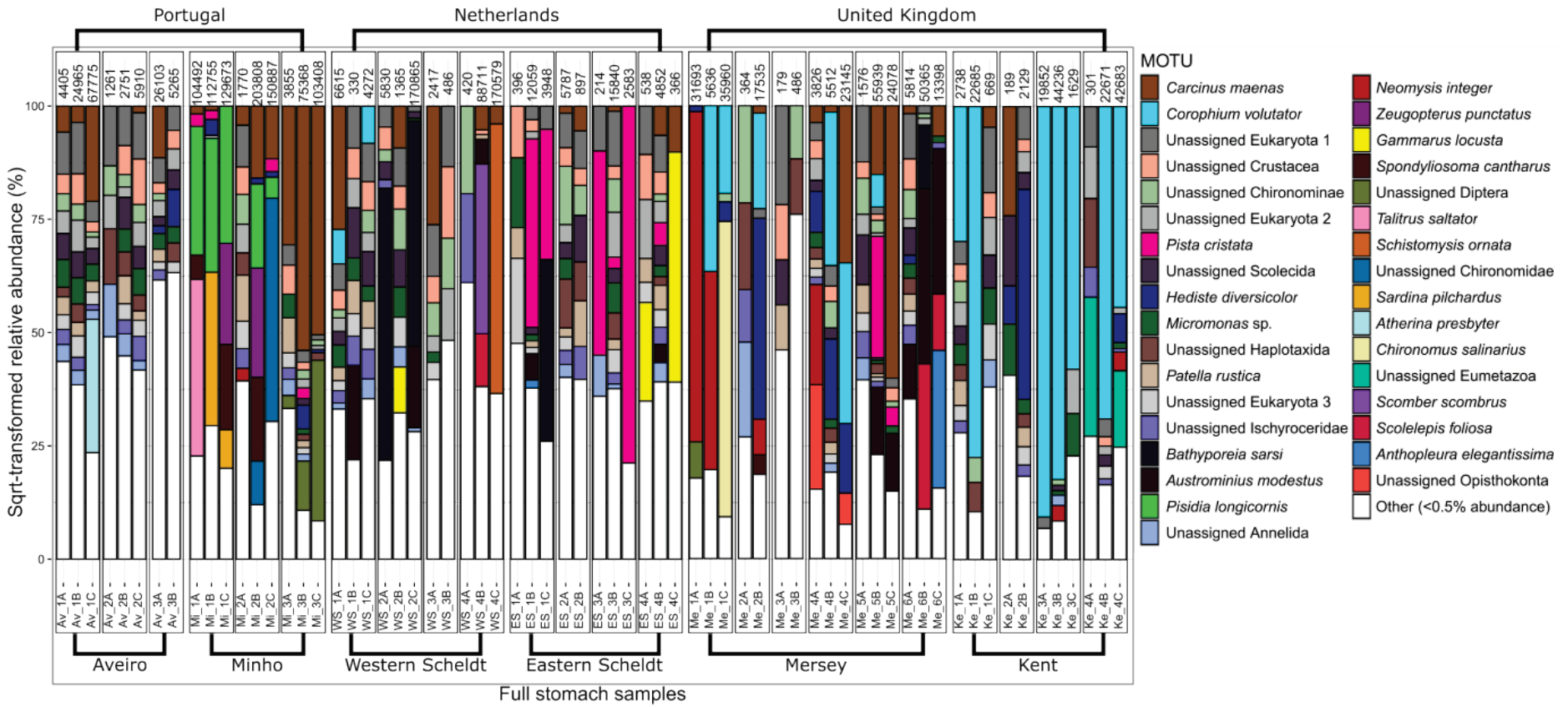

Figure 4.2. Relative abundances (square root-transformed) of MOTUs detected in *Crangon crangon* stomach samples by COI metabarcoding. Each bar represents one sample. Countries are shown on top of the graph, estuaries below and boxes contain the individual sites. The number on top of each sample represents the number of COI reads. The category other is comprised of MOTUs with < 0.5% COI reads. Sqrt: square root.

Table 4.1. Trophic significance of *Crangon crangon* prey items. MOTUs shown (≥5% relative abundance in the stomach samples) are assigned to the family level or lower. Best identity: the best match (in proportion) to the query sequence found in the reference database. Trophic significance: See text. (overleaf).

Phylum	Order	Family	Species	Best identity	Presence (%)	Mean (±SE) Abundance (%)	Mean (±SE) Selectivity (D)	Trophic significance	Literature	Source
Annelida	Phyllodocida	Nereididae	*Hediste diversicolor*	0.98	25.8	3.7±2.2	0.7±0.1	Medium	Sto+	Lloyd and Yonge (1947); Pihl and Rosenberg (1984)
	Spionida	Spionidae	*Scolelepis foliosa*	1.00	4.5	0.7±0.6	1.0±0.0	Low	Exp[3] Sto-[3]	Van Tomme et al. (2014); Ansell et al. (1999)
	Terebellida	Terebellidae	*Pista cristata*	0.99	18.2	3.8±2.4	0.6±0.1	Medium	Sto-[3]	Ansell et al. (1999)
Arthropoda	**Amphipoda**	**Corophiidae**	***Corophium volutator***	**0.99**	**22.7**	**10.7±4.8**	**1.0±0.0**	**High**	Sto+	Pihl and Rosenberg (1984); Evans (1984)
		Gammaridae	*Gammarus locusta*	1.00	4.5	1.2±1.2	1.0±0.0	Low	Sto+	Plagmann (1939)
		Ischyroceridae	Unassigned	0.94	42.4	0.6±0.2	0.6±0.1	Medium		
		Pontoporeiidae	*Bathyporeia sarsi*	1.00	6.1	3.0±2.2	1.0±0.0	Medium	Exp	Van Tomme et al. (2014)
		Talitridae	*Talitrus saltator*	0.99	1.5	0.7±0.7	1.0±0.0	Low		
	Decapoda	**Carcinidae**	***Carcinus maenas***	**1.00**	**50.0**	**7.2±3.0**	**1.0±0.0**	**High**	Exp[1] Sto-	Moksnes et al. (1998); Raffaelli et al. (1989); Pihl and Rosenberg (1984)
		Porcellanidae	*Pisidia longicornis*	1.00	7.6	1.7±1.4	1.0±0.0	Medium		
	Diptera	Chironomidae	Unassigned	0.88	50.0	1.3±0.5	0.8±0.1	Medium	CA	Nordström et al. (2009)
		Chironomidae	*Chironomus salinarius*	1.00	1.5	1.2±1.2	1.0±0.0	Low		
	Mysida	Mysidae	*Neomysis integer*	0.98	10.6	2.6±2.1	1.0±0.0	Medium	Sto-	Raffaelli et al. (1989)
		Mysidae	*Schistomysis ornata*	0.98	1.5	1.0±1.0	1.0±0.0	Low	Sto+[3]	Oh et al. (2001)
	Sessilia	Austrobalanidae	*Austrominius modestus*	1.00	15.2	2.3±1.5	1.0±0.0	Medium		
Chordata	Atheriniformes	Atherinidae	*Atherina presbyter*	1.00	1.5	0.6±0.6	1.0±0.0	Low		
	Clupeiformes	Clupeidae	*Sardina pilchardus*	1.00	3.0	0.6±0.6	1.0±0.0	Low		
	Pleuronectiformes	Scophthalmidae	*Zeugopterus punctatus*	0.99	3.0	0.8±0.5	1.0±0.0	Low		
	Scombriformes	Scombridae	*Scomber scombrus*	1.00	1.5	0.6±0.6	1.0±0.0	Low		
	Spariformes	Sparidae	*Spondyliosoma cantharus*	1.00	6.1	0.5±0.3	1.0±0.0	Low		
Other*	Mamiellales	Mamiellaceae	*Micromonas sp.*	0.99	50.0	0.6±0.2	0.5±0.1	Medium		
	Actiniaria	Actiniidae	*Anthopleura elegantissima*	0.99	3.0	0.5±0.5	1.0±0.0	Low		
	Patellogastropoda	Patellidae	*Patella rustica*	1.00	42.4	0.6±0.2	0.7±0.1	Medium		

Other*: Chlorophyta; Cnidaria; Mollusca; Exp: Experimental study; Sto+: Major contributor based on stomach analysis; Sto-: Minor contributor based on stomach analysis; CA: Contribution assumed by source; [1]: Larvae; [2]: Adults; [3]: Related taxa (same family). In bold: High trophic significant taxa

MOTU diversity within phyla was generally higher in the sediment than in the stomach samples, with the exception of Arthropoda, Annelida, Mollusca and Chordata (Figure 4.4A). The proportion of MOTUs that could not be assigned to the phylum level was higher in the sediment (73%) than in the stomach samples (57%). Empty stomach samples contained a very low number of MOTUs and reads, and were, therefore, not taken into account for any further analyses. Data combined per sample type (sediment/stomach) and phylum showed

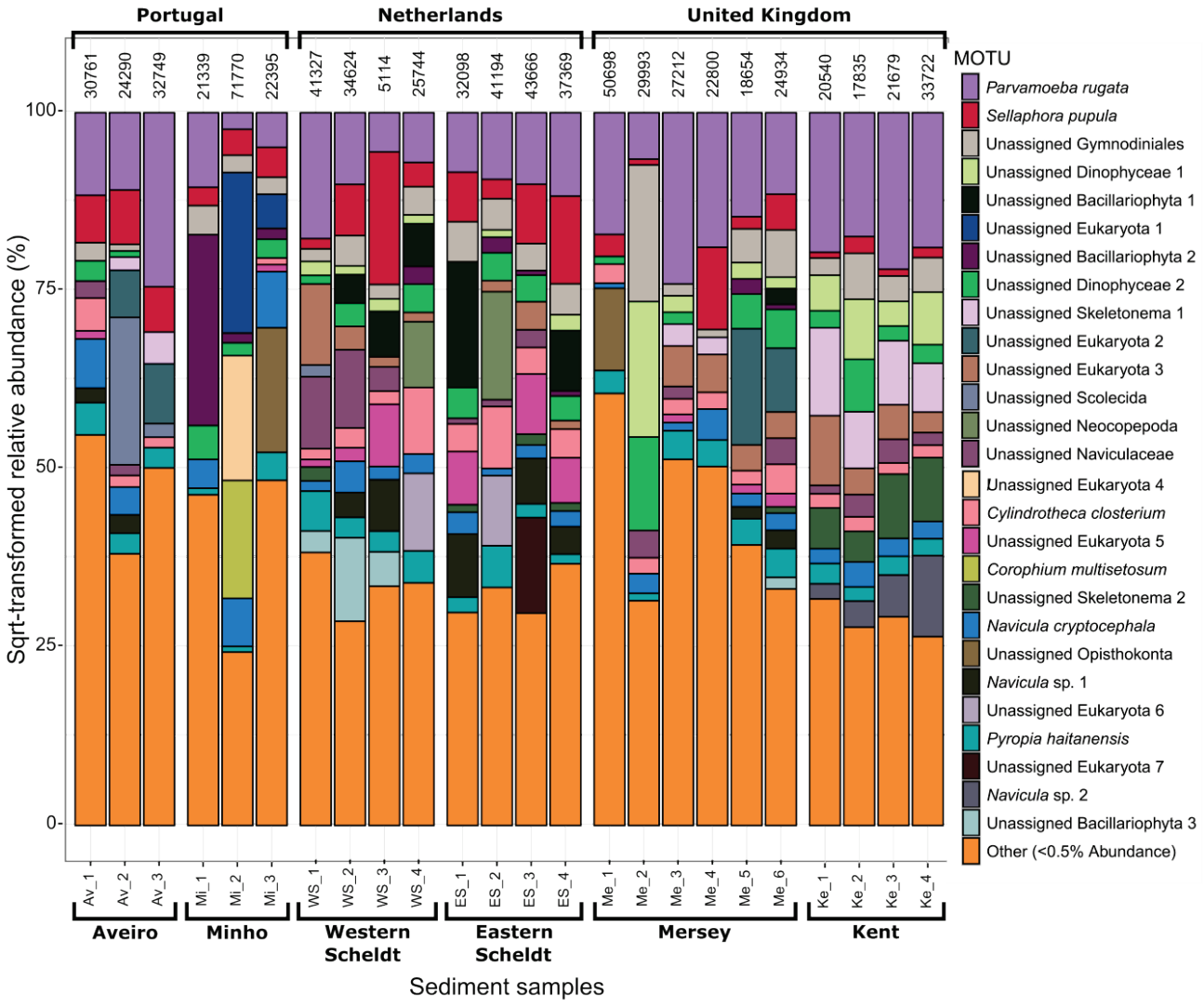

Figure 4.3. Relative abundances (square root-transformed) of MOTUs detected in sediment samples by COI metabarcoding. Each bar represents one sample. Countries are shown on top of the graph, estuaries below. The number on top of each sample represents the number of COI reads. The category other is comprised of MOTUs with < 0.5% COI reads. Sqrt: square root.

that sediment samples contained high relative read abundances of Bacillariophyta (20±3%), Discosea (11±2%), Dinoflagellata (6±2%) and Arthropoda (5±2%) while *C. crangon* stomach samples contained a high mean (±SE) relative read abundance (%) for Arthropoda (47±4%), Annelida (13±3%) and Chordata (5±2%; Figure 4.4B). PERMANOVA analysis at the phylum level showed significant differences between sediment and full stomach samples (pseudo-F = 34.7, P < 0.001). Apart from Cnidaria and Rhodophyta, all phyla with ≥ 1% abundance in either sediment or stomach samples showed significant differences (based on paired Wilcoxon signed-rank tests) in relative read abundances between the sediment and stomach samples (Appendix 4.5). Visualisation of the importance of the phyla detected in the stomach samples based on the mean relative abundance (%), presence (%) and Jacobs' selectivity index (D) is shown in Figure 4.4C.

4.4.4. Variation between estuaries

Analysis of DNA extracted from both sediment and *C. crangon* pooled stomach samples showed significant differences between sample types (Figures 4.5 and 4.6A; PERMANOVA: pseudo-F: 8.2, P < 0.001) and estuaries (Figure 4.5; PERMANOVA: pseudo-F: 2.2, P< 0.001). MOTUs abundant in the stomach samples (≥ 0.5% abundance) showed a low read abundance in the sediment samples from all estuaries (Figure 4.6A). Multivariate analysis on stomach contents only showed significant differences between estuaries (Figure 4.6B; PERMANOVA: pseudo-F: 2.1, P < 0.001) and sites nested within estuaries (PERMANOVA: pseudo-F: 0.5, P < 0.001). Bonferroni-corrected pairwise comparisons showed that, apart from the Portuguese Minho and Aveiro estuaries, stomach communities of adjacent estuaries were not significantly different. Significant differences were found in stomach contents from several non-adjacent estuaries (See appendix 4.6 for details).

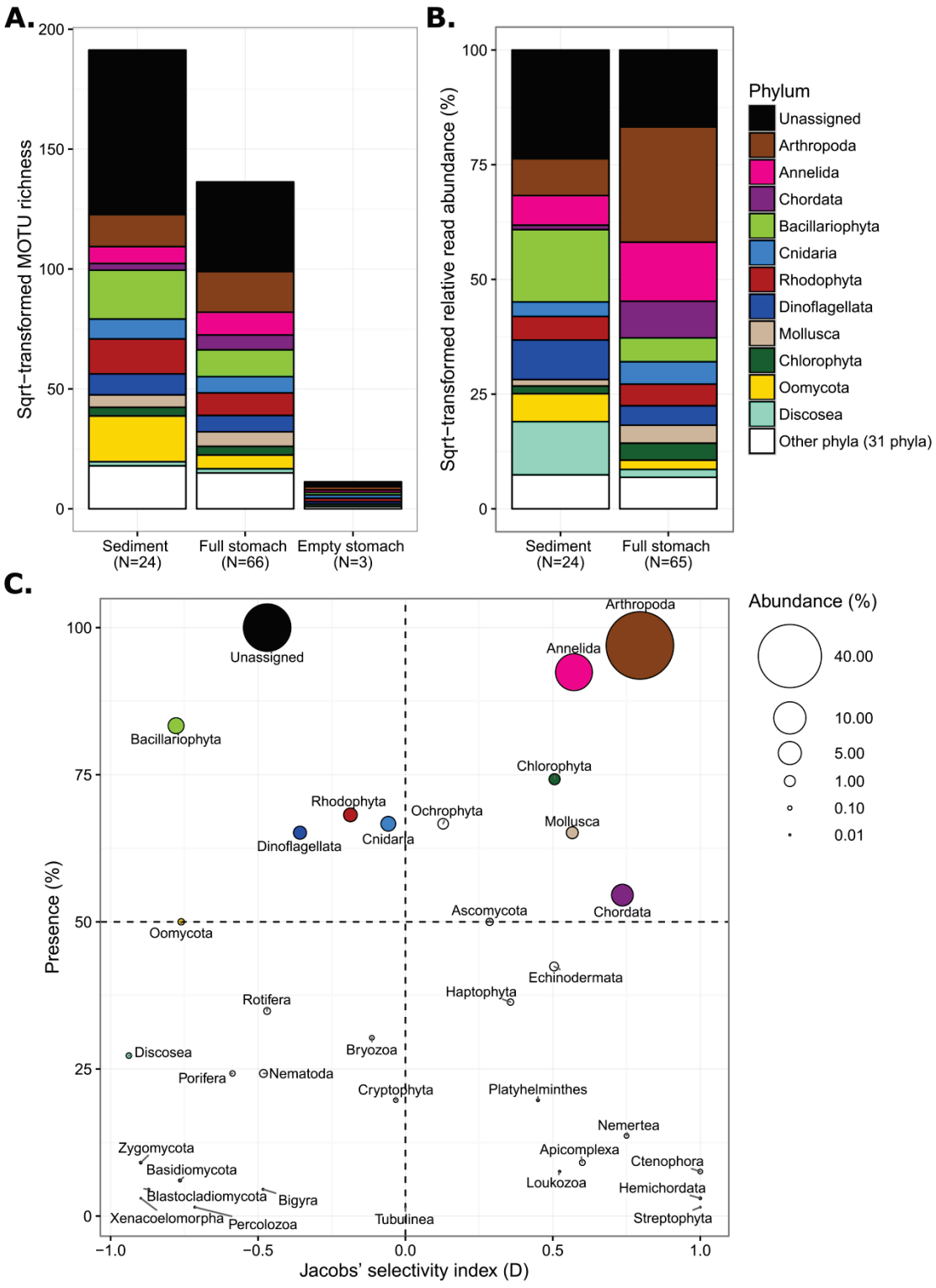

Figure 4.4. Phyla detected in sediment and *Crangon crangon* stomach samples by COI metabarcoding. (A) Total number of MOTU (square root transformed) detected per phylum in sediment, full stomachs and visually empty stomachs. (B) Mean relative read abundance (square root transformed) of phyla detected in sediment and *C. crangon* full stomach samples. (C) Phylum trophic significance based on presence (%), mean relative abundance (%) in full stomach samples and Jacobs' selectivity index. Stomach samples consisted of a pool of up to 8 stomachs. The category "other phyla" (represented in white) contains phyla with < 1% COI reads in both the sediment and full stomach samples. Sqrt: square root.

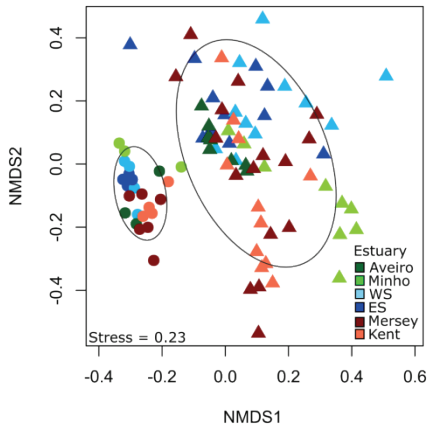

Figure 4.5. Multidimensional scaling analysis of MOTUs detected in sediment (dots) and *Crangon crangon* stomach samples (triangles), based on square-root transformed Bray-Curtis dissimilarities. 75% confidence ellipses are shown per sample type. WS = Western Scheldt; ES = Eastern Scheldt.

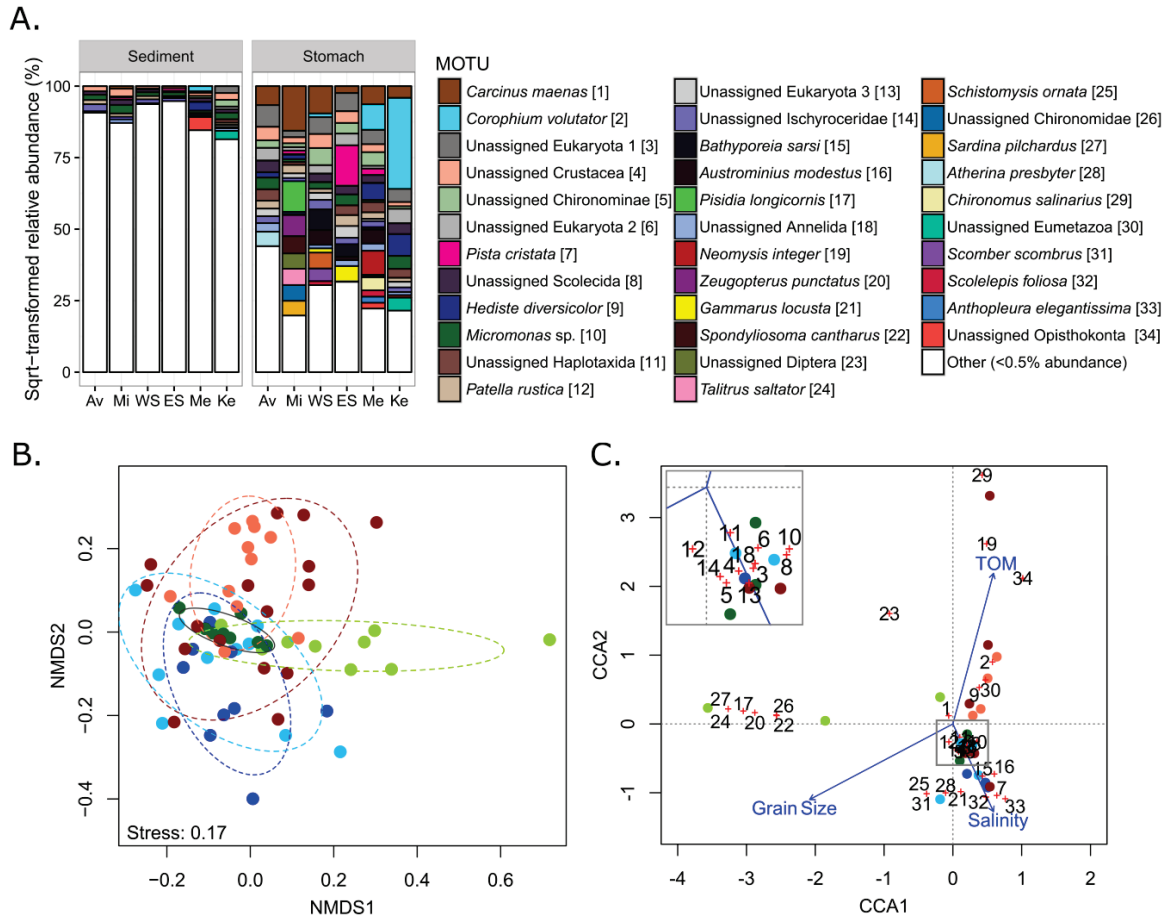

Figure 4.6. Multivariate analysis of *Crangon crangon* diet in six estuaries determined by COI metabarcoding based on MOTUs (N = 34) over all stomach samples (N = 66). (A) Mean relative read abundance of each MOTU per estuary based on DNA extracted from sediment and stomach samples. "Other" comprises of MOTU < 0.5% mean abundance in the stomach samples. MOTUs are identified for ≥ 0.5% average read abundance in the stomach samples, otherwise are referred as "Other" (B) Multidimensional scaling (nMDS) analysis based on square-root transformed Bray-Curtis dissimilarities of *C. crangon* stomach samples. Each dot represents one pooled stomach sample, estuaries are identified by colours (see below) and ellipses show 75% confidence intervals. (C) Canonical correspondence analysis (CCA) of square-root transformed relative read abundances in relation to salinity, total organic matter (TOM) and median grain size. Reads were averaged per site (displayed as dots) and estuaries are identified by colour (see below). Red crosses represent the MOTU scores and numbers refer to the MOTU names given in panel A. The inset zooms in on the centre of the graph. Estuaries: Dark green = Aveiro (Av); light green = Minho (Mi); light blue = Western Scheldt (WS); dark blue = Eastern Scheldt (ES); dark red = Mersey (Me); light red = Kent (Ke).

Step-wise model selection (both forward and reverse) and CCA (Figure 4.6C) showed significant influences of salinity ($P < 0.01$), median grain size ($P < 0.01$) and TOM ($P < 0.05$; see appendix 4.7 for means per estuary) on MOTU composition in *C. crangon* stomach samples (≥ 0.5% abundant MOTUs). The environmental variables (constrained CCA axes) explained 29% of the variance in the data set. Temperature, turbidity and oxygen saturation did not have a significant influence on the model and pH was strongly correlated with salinity ($r^2 = 0.73$, $P < 0.001$, $N = 24$). These factors were, therefore, not included in the final model. MOTU richness (rarefied to 250 reads) in *C. crangon* stomach contents also showed differences between estuaries, with the Aveiro and Western Scheldt estuaries showing a higher number of MOTUs than the others (Figure 4.7). The slopes of the MOTU accumulation curves, however, did not approach an asymptote, offering a glimpse of the vast amount of marine biodiversity yet to be uncovered.

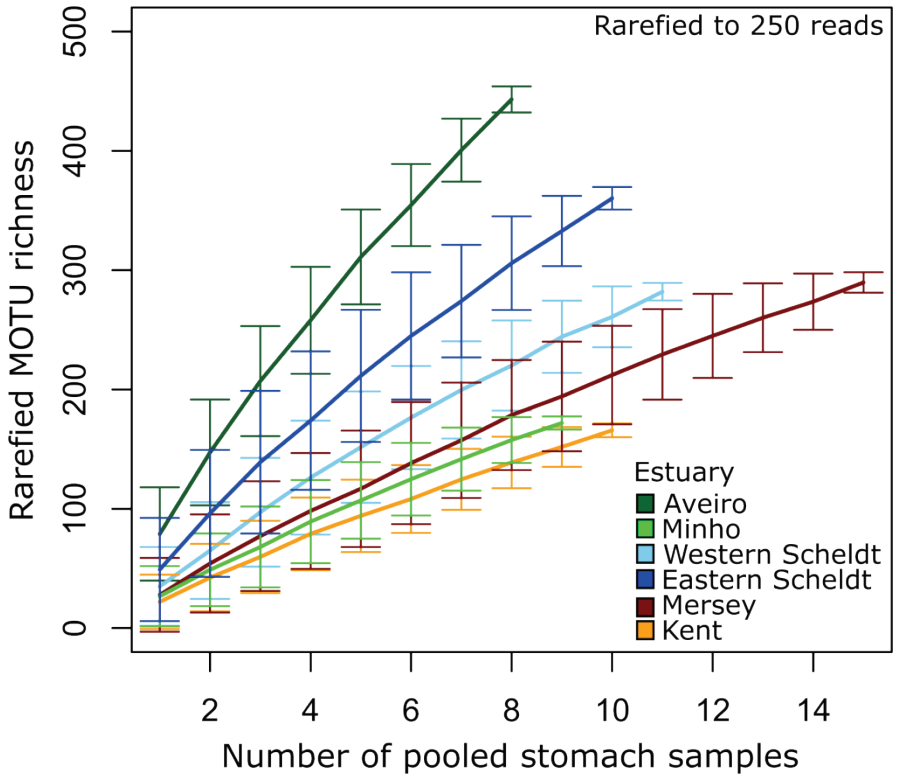

Figure 4.7. MOTU accumulation curves showing MOTU richness (based on all MOTUs detected) in *Crangon crangon* pooled stomach samples in several European estuaries. Each sample has been rarefied to 250 reads prior to the construction of the accumulation curves.

4.5. Discussion

4.5.1. Evaluation of *C. crangon* diet

Coastal and estuarine habitats are important hotspots for biodiversity and highly productive ecosystems. At the interface between terrestrial and aquatic environments, they provide essential ecosystem services and functions, from carbon and nutrient cycling, to energy exchange. They also represent nursery grounds for ecologically and economically important species (Martínez et al., 2007, Hyndes et al., 2014, Sheaves et al., 2015), making the understanding of biodiversity and trophic interactions a key step towards long-term ecosystem management (Pimm et al., 1991, Pihl, 1985). The brown shrimp is a key component of European sandy shores (Evans, 1984, Campos and van der Veer, 2008), where it is an essential prey for many commercial fish species (Hostens and Mees, 1999, Henderson et al., 1992) and has a fundamental role as an opportunistic/generalist predator. This study provides a detailed overview of its trophic ecology, focusing on dietary variations at multiple geographical scales.

Adult brown shrimp (approx. 35 mm TL) were caught in a variety of sandy estuarine intertidal habitats. Mainly females were captured, probably due to the spatial sex-specific segregation of *C. crangon* during the summer-autumn period (Henderson and Holmes, 1987, Bamber and Henderson, 1994). The results confirm *C. crangon* as a generalist consumer feeding on a broad variety of food items (Evans, 1983, Pihl and Rosenberg, 1984), but preferring arthropods, annelids and fish. Several studies concluded that arthropods and annelids were its main prey categories (Oh et al., 2001, Ansell et al., 1999, Plagmann, 1939), but the relative abundance of decapod and fish detected in this study was higher than in other studies (e.g. Oh et al., 2001, Ansell et al., 1999, Raffaelli et al., 1989). Crangonid shrimp often eat only the soft body parts of these large prey (Gibson et al., 1995, Seikai et al., 1993) and macerate them to a fine degree (Asahida et al., 1997, Wilcox and Jeffries, 1974). Smaller food items, on the other hand, are often ingested as a whole, including their hard body parts (Tiews, 1970) and are thus more easily identified by morphological methods (Barrett et al., 2007). This discrepancy in detectability might possibly have played a factor in studies that detect low amount of fish and decapods but high amounts of unidentified soft tissue (e.g. Oh et al., 2001, Raffaelli et al., 1989). Metabarcoding methods can detect and taxonomically identify such soft tissues, thus highlighting the enhanced suitability of genetic approaches to offer a more realistic picture of trophic ecology in marine invertebrates.

The diet of *C. crangon* showed a high MOTU richness, including previously described food items (Table 4.1). The number of COI MOTUs (2429) detected in the shrimp's stomachs may be an overestimation of the total number of real species (e.g. due to detection of pseudogenes; Tang et al., 2012, Vamos et al., 2017), yet, even just the 306 ascertained species in the shrimp's diet was remarkably higher than ever found in any previous study based on morphological identification (see Table 4.1). Two species were predominant in our study: *C. maenas* across the overall geographic distribution and *C. volutator* in UK localities (characterised by muddy sediments and high organic matter content). Both species are well-known prey of *C. crangon* (Moksnes et al., 1998, Pihl and Rosenberg, 1984, Evans, 1984) and can occur at high densities in soft-bottom habitats (Moksnes, 2002, Meadows and Reid, 1966). Consumption of *C. maenas* could be the result of scavenging, although juvenile crabs could be captured, while *C. volutator* is likely to be predated, as these amphipods are small. Overall, the local distribution of the detected food items followed environmental gradients reflecting their ecology. Euryhaline deposit feeders such as *C. volutator* and *H. diversicolor, N. integer* and *Chironomus salinarius* (probably larvae) were mainly associated with muddy, brackish sites with high organic matter content, which these species commonly inhabit (Mees et al., 1993, Mauchline, 1971, Meadows, 1964, Ólafsson and Persson, 1986, Anderson, 1972, Drake and Arias, 1995). Stomach samples taken from sites with high grain size contained species adapted to coarser sands, such as *P. longicornis* and *Talitrus saltator* (Fanini et al., 2007, Pallas et al., 2006). The variety of fish species detected indicates a combination of direct predation on juveniles of species which use the estuaries as nurseries (e.g., *Platichthys flesus* and *Dicentrarchus labrax*) and scavenging on dead bodies of species which do not regularly use estuaries as a nursery (e.g., *Scomber scombrus* and *Labrus bergylta*; Elliott and Dewailly, 1995) . The high presence of the invasive barnacle *A. modestus* DNA at several locations was likely due to the capture of cypris or nauplii larvae (Boddeke et al., 1986, Ansell et al., 1999) since the adults prefer hard substrates (Moore, 1944). The picoplanktonic algae *Micromonas* sp. was detected frequently but, due to its small size, it is unlikely to be actively consumed by shrimp. This species is a major component of the planktonic community (Not et al., 2004) and possibly passively ingested directly from the water column or obtained by means of secondary predation (Loo et al., 1993, Perissinotto and McQuaid, 1990).

This is also the first study showing a high occurrence of *P. lilacinum* (Ascomycota: Hypocreales) in the digestive system of *C. crangon*. *Purpureocillium lilacinum* is a well-studied fungus, being abundant in terrestrial soils (Cham Thi Mai et al., 2016) and detected in the marine environment (Yue et al., 2015, Redou et al., 2015). It is a known pathogen of nematodes and therefore of commercial importance as a biological control agent to manage pests of several crops (Castillo Lopez et al., 2014, Singh et al., 2013). This fungus is even considered to be of medical importance since it can infect humans and other vertebrates with compromised immune systems (Luangsa-Ard et al., 2011). As *P. lilacinum* grows well on marine shrimp tissue (*Penaeus* sp.; Nidheesh et al., 2015) and is closely related to known parasites of crabs (Smith et al., 2013), it might be postulated that it has a parasitic relationship with *C. crangon*. More research is required to test this hypothesis. Its present occurrence and high relative abundance (although possibly overestimated since its DNA was extracted from a living community, as opposed to digested food) in *C. crangon* stomach samples over a large geographical area are clear indicators that this species might be important for the brown shrimp's ecology and/or physiology.

4.5.2. The application of metabarcoding in crustacean trophic studies

The results show that metabarcoding is a powerful tool to study the trophic ecology of generalist consumers (Leray et al., 2015, Pompanon et al., 2012, Kartzinel and Pringle, 2015). The versatile primers used during this study allowed for the detection of a wide range of taxa with a high taxonomic resolution (see Wangensteen et al. in review for more information) and the amplification of digested and highly decomposed DNA (Berry et al., 2015). Moreover, even without the use of blocking primers, the fraction of the brown shrimp DNA detected in its own gut was low (average: 28%; compared to e.g. Pinol et al., 2014, Olmos-Perez et al., 2017). Blocking primers are often used in trophic metabarcoding studies to avoid amplification of the DNA of the subject of the study (e.g. Ray et al., 2016, Xiong et al., 2017), but they can co-block the DNA of specific prey species (Pinol et al., 2015, Pinol et al., 2014).

This study clearly confirms that metabarcoding using universal primers without predator-specific blocking primers is a simple, rapid and relatively inexpensive method to define in detail the feeding ecology of organisms (Pinol et al., 2014, Berry et al., 2015, Kartzinel and

Pringle, 2015). Metabarcoding has several clear advantages over traditional trophic methods including the better detection of soft-bodied, small and cryptic taxa, higher speed of analysis (Chariton et al., 2015, Berry et al., 2015, Casper et al., 2007, Symondson, 2002), enhanced objectivity and traceability of identifications, which do not rely on the availability of morphological taxonomic expertise.

Although the DNA extracted from visually empty *C. crangon* stomachs was too low in prey read number and MOTU diversity to be compared with full stomach samples, we showed that prey DNA can be detected even in empty guts (Harms-Tuohy et al., 2016). About 40% *C. crangon* caught had an empty stomach (in line with: Raffaelli et al., 1989, Feller, 2006, Pihl and Rosenberg, 1984), but no correlation was found between the fraction of full stomachs and the time of day (in contrast to: Feller, 2006, Pihl and Rosenberg, 1984) due to the lack of samples collected at night. The opportunity to obtain feeding habits information even from empty stomachs can be of importance, for instance, when studying predators which do not often have full stomachs (Arrington et al., 2002, Satoh et al., 2004, Olatunde, 1978).

Both traditional morphological examination and DNA-metabarcoding of food items suffer from limitations in providing quantitative descriptions of the diet of consumers (Casper et al., 2007). For metabarcoding, errors can occur due to technical artefacts specific to DNA amplification and sequencing (Barnes and Turner, 2016, Pompanon et al., 2012), and biological limitations such as species-specific digestion and DNA degradation rates (Deagle et al., 2010, Sakaguchi et al., 2017, Pinol et al., 2014, Murray et al., 2011). Furthermore, not all DNA detected might come from directly eaten species. Secondary predation (taxa present in the stomach of preyed organisms) is a recognised issue in metabarcoding studies (Kartzinel and Pringle, 2015, Berry et al., 2015), and possibly part of the reads detected in our study might have been the result of secondary predation. Cannibalism also imposes a specific problem in trophic molecular studies since it cannot be identified by means of metabarcoding (Berry et al., 2015, Ray et al., 2016). Large brown shrimp*s* are known to be cannibalistic (Pihl and Rosenberg, 1984, Evans, 1984) but the removal of *C. crangon* sequence reads from our data set makes it impossible to gauge insights into the extent of cannibalism in this species. Due to the restrictions in the quantification of consumed prey volume, many trophic studies only use presence/absence data (e.g. Pinol et al., 2015, Deagle et al., 2010, Harms-Tuohy et al., 2016). This might, however, result in an overestimation of small taxa that are abundant in the sediment, but with low trophic relevance, as they could,

in the case of *C. crangon*, be passively acquired when shrimp ingest sediment to crush food in their stomach (Tiews, 1970, Ansell et al., 1999). Multiple stomachs were pooled prior to analysis and data was subjected to rigorous filtering to allow for a semi-quantitative estimation of proportions of prey DNA (Deagle et al., 2005, Pompanon et al., 2012, Thomas et al., 2016, Lejzerowicz et al., 2015). Relative abundances of individual taxa should, however, be considered with caution and viewed more in categorical terms (low or high trophic significance) than exact proportions (Deagle et al., 2005). This study provides a significant addition to a growing body of studies in showing the applicability of semi-quantitative estimations in molecular trophic ecology (e.g. Sakaguchi et al., 2017, Soininen et al., 2013, Albaina et al., 2016, Ray et al., 2016).

4.5.3. Geographic variation in *C. crangon* trophic ecology

This is the first study showing large geographical variation in the brown shrimp's trophic ecology at multiple spatial scales. Previous studies have shown local variability in *C. crangon* diet (Pihl and Rosenberg, 1984, Evans, 1984, Oh et al., 2001) but no studies have been performed across multiple European estuaries. The results indicate that the consumed prey community can vary at local (within estuary, as discussed above) and regional (between estuaries) scales. The use of molecular markers to study local variation in diet of a species capable of seasonal and tidal migrations (Al-Adhub and Naylor, 1975, Henderson and Holmes, 1987) is possible due to its relatively fast gut passage time (4-20h; Feller, 2006, Pihl and Rosenberg, 1984, van der Veer and Bergman, 1987). Large scale assessment of *C. crangon*'s trophic ecology showed differences among distant estuaries and similarities among adjacent estuaries, with the exception of the Minho and the Aveiro estuaries, which are geographically close but significantly different in biodiversity and thus in *C. crangon* stomach contents. The former is characterized by high water discharge and salinity variations (Costa-Dias et al., 2010), resulting in a low biodiversity while the latter forms a large, saline lagoon with a wide variety of different habitats incorporating euhaline/polyhaline areas with relatively high species richness (Rodrigues et al., 2011). Since Aveiro is located in the most southern range of the study area, latitude might also play a role in the high species richness detected (Attrill et al., 2001). Other estuaries characterized by lower species richness in the stomach contents of *C. crangon* were the Mersey estuary, which has a history of anthropogenic stress (Jones, 2000), and the Kent estuary, which is

characterised by fine sediments and low salinity (Anderson, 1972). Overall, trophic variation in *C. crangon* depends on patterns in the local abundance and distribution of its prey (in line with: Oh et al., 2001, Pihl and Rosenberg, 1984, Pihl, 1985). In order to evaluate this variation, knowledge on the ecology and seasonality of the local macrozoobenthic community is required.

4.5.4. Crangon crangon's ecological role

What is the true ecological role of adult brown shrimp in European estuaries? Based on the results of this study, *C. crangon* can best be described as a highly opportunistic carnivore. Its flexible trophic ecology might contribute to its very wide distribution on European coasts (Campos et al., 2009b). In order to feed on diverse prey taxa, adult *C. crangon* are capable of employing a variety of methods including ambush predation (Gibson et al., 1995, Pinn and Ansell, 1993), gulping behaviour (Tiews, 1970) and scavenging (Figure 4.8; Price, 1962, Ansell et al., 1999). Since meiofaunal and protist phyla were in general negatively selected on (present but not abundant; in line with: Feller, 2006, Evans, 1983), it is possible that these taxa were passively consumed during the ingestion of sand to aid digestion (Tiews, 1970, Ansell et al., 1999) or through secondary predation. Larger prey species, such as decapods and fish, are likely caught as juveniles or eggs by ambush predation (Gibson et al., 1995, Oh et al., 2001, Pihl and Rosenberg, 1984, van der Veer and Bergman, 1987) or consumed as adults by scavenging (Ansell et al., 1999, Price, 1962). Carcasses might be encountered during tidal migrations or at night, when shrimp are more active (Al-Adhub and Naylor, 1975, Pihl and Rosenberg, 1984, del Norte-Campos and Temming, 1994). Several studies classify *C. crangon* as an omnivore (Raffaelli et al., 1989, Tiews, 1970, Lloyd and Yonge, 1947, Ansell et al., 1999), but we cannot confirm this classification, because the primers used during this study have a very low affinity for chlorophytes resulting in many algal taxa not being detected (Wangensteen et al. in review). Nevertheless, the algal phyla that can be detected with these primers (e.g. Rhodophyta, Phaeophyta and Bacillariophyta) had a low selectivity, indicating a negligible trophic importance for *C. crangon*. More research is required with plant-specific primers to assess the actual contribution of herbivory to the diet of the brown shrimp.

4.5.5. Conclusions

Several studies show that crustaceans such as *C. crangon* can have a profound impact on local communities (Pihl and Rosenberg, 1984, Van Tomme et al., 2014, Evans, 1984). Due to its high year-round abundance (Bamber and Henderson, 1994, Pihl and Rosenberg, 1984) and the wide range of prey items ingested, the brown shrimp can be considered as a dominant carnivore in European estuarine sandy habitats (Amara and Paul, 2003, Kuipers and Dapper, 1981). The results of this study also confirm that trophic metabarcoding of crustacean consumers is of relevance for other biological fields such as parasitology and invasion biology since it allows for the detection of known and newly discovered parasites (eg. *Hematodinium* sp., Apicomplexa and the fungus *P. lilacinus*; Molnar et al., 2012; Rueckert et al., 2011; Stentiford and Shields, 2005) and alien species (Austrominius modestus; Gallagher et al., 2015) . Furthermore, the consumption of ecosystem engineers such as *H. diversicolor* might influence the nutrient recycling in estuarine ecosystems (Raffaelli, 2006). The brown shrimp is also an essential prey item for many species, including birds and fish, representing a key component of the soft-bottom food web (Hostens and Mees, 1999, Evans, 1984, Bamber and Henderson, 1994, Walter and Becker, 1997). Given its important role in the ecosystem, its commercial importance and its influence as a model organism for behavioural, physiological, ecotoxicological, camouflage and community dynamic studies (e.g. Hunter et al., 1998; Chapters 2 & 3, Brown, 1946, Hedvall et al., 1998, Hagerman and Szaniawska, 1986) a detailed knowledge of its diet is required. The wide taxonomic breadth of the brown shrimp's diet together with the high prevalence of soft tissue and endoparasitic fungi in its stomach contents allows for the application of metabarcoding to its fullest extent in order to provide a holistic interpretation of the trophic ecology of this highly adaptive predator and, thereby, inferring essential insights in community interactions on estuarine soft bottom habitats.

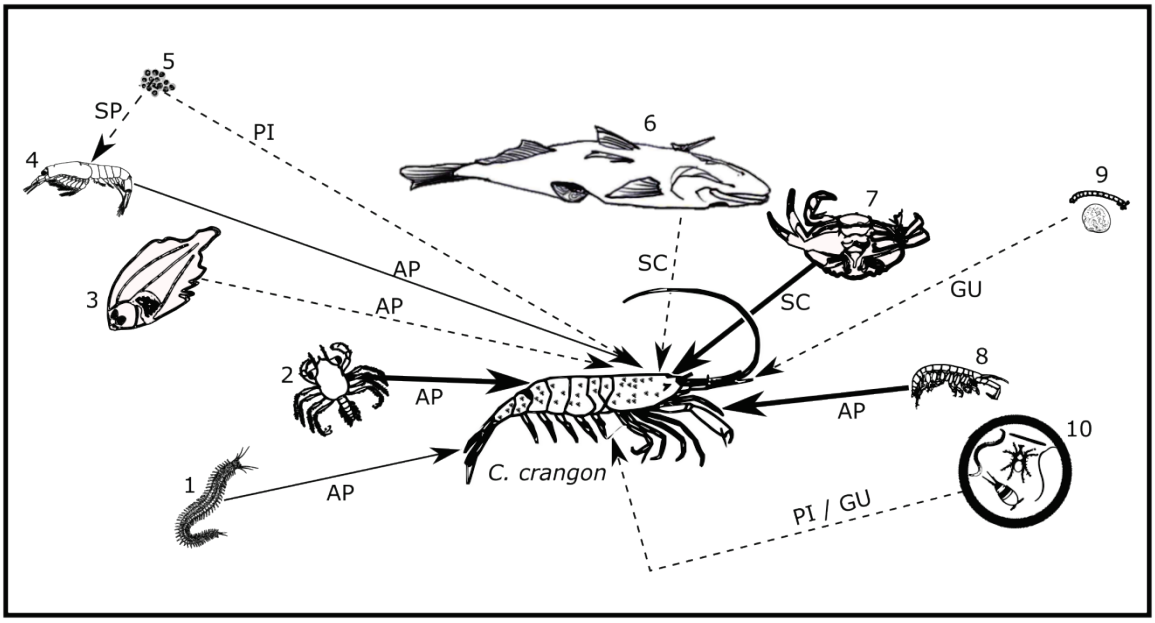

Figure 4.8. Schematic representation of the most important food items of adult *Crangon crangon* and their probable method of capture/ingestion. Line thickness represents trophic significance: high (bold); medium (thin); low (dashed). Numbers identify prey categories: annelids (1); decapod larvae/instars (2); fish 0-year-juveniles (3); 4 mysids (4); (pico) phytoplankton (5); fish carcasses (6); decapod carcasses (7); amphipods (8); chironomid, mollusc and barnacle larvae (9); meiofauna (10). Letters define method of ingestion: Secondary predation (SP); ambush predation (AP); gulping predation (GU); passive ingestion (PI); scavenging (SC). Images not to scale.

Chapter 5.

The application of trophic molecular ecotoxicology in the brown shrimp, *Crangon crangon*, for environmental assessments in European estuaries

5.1. Abstract

Environmental monitoring of estuarine waters requires accurate and reliable estimates of benthic diversity and a comprehensive understanding of species interactions. Molecular tools, such as metabarcoding, have the advantage over traditional biodiversity and trophic interaction monitoring tools due to their ability to detect small, cryptic and digested species with reduced effort and high precision. Metabarcoding has, however, not yet been applied to assess the impact of heavy metal pollution on benthic communities and trophic interactions in estuarine systems. Here, I tested the application of metabarcoding to study variation in estuarine benthic communities and diet of the brown shrimp *Crangon crangon* L., an abundant opportunist in European estuaries, in relation to heavy metal pollution. Results, obtained from six European estuaries, showed no variation in benthic community structure and *C. crangon*'s diet between impacted and reference estuaries, and no correlation between heavy metal concentrations and estuarine benthic community structure. Significant relationships were detected between variation in *C. crangon*'s diet and bioaccumulated heavy metal concentrations for some heavy metal elements (especially copper). Also, the relative abundance of a potentially endoparasitic fungus (*Purpureocillium lilacium*) detected in the shrimp's stomachs was possibly influenced by bioaccumulated copper levels. The integration of molecular trophic information in ecotoxicological studies can reveal essential information on the effects heavy metal pollution on species interactions such as predation and parasitism. The results of this chapter may contribute to future ecotoxicological research and are a first, but significant, step to the inclusion of metabarcoding studies on the gut contents of opportunistic predators in environmental assessments.

5.2. Introduction

Estuaries are under global anthropogenic pressure, which results in stress on their important functions as biodiversity hotspots and nursery grounds for ecologically and economically important species, and might affect the ecosystems services they provide (Halpern et al., 2008, Worm et al., 2006, Scheffer et al., 2005, Sheaves et al., 2015). Of these human impacts, heavy metals are of special concern due to their rapid increase during recent centuries and tendency to accumulate in bottom sediments (Ansari et al., 2004, Dauvin, 2008, Yang and Rose, 2005, Spencer et al., 2003). Many heavy metal contaminants can accumulate through the food web and are known to disrupt important physiological functions and behaviours (Barata et al., 2004, Scott and Sloman, 2004, Szaniawska, 1985, Blaxter and Hallers-Tjabbes, 1992). Impacts of heavy metals on animals range from the cellular to the individual and population level (Galloway et al., 2004, Santos et al., 2000, Sánchez-Moreno and Navas, 2007). Some heavy metals can have endocrine disrupting effects by disrupting the synthesis and excretion of hormones, interfering with hormone-receptor interactions, or disrupting the synthesis of hormone receptors. This can result in, for example, intersex individuals and inhibited moulting patterns (Scott and Sloman, 2004, Rodríguez et al., 2007, Clotfelter et al., 2004). Pollutants can also influence the fitness of animals at sublethal levels by affecting the balance between the organism and its environment and even prove to be lethal in the long term (Blaxter and Hallers-Tjabbes, 1992). Even more, these contaminants can interact with each other and natural stressors, resulting often in synergistic effects (Crain et al., 2008, Holmstrup et al., 2010, Coors and De Meester, 2008).

The sensitivity of marine animals to heavy metal contamination depends on a variety of factors, including their rate of accumulation and depuration (Ansari et al., 2004, Hunter et al., 1998, White and Rainbow, 1982, Ray et al., 1981). Some animals are very sensitive to low heavy metal levels while others can deal with high concentrations in the environment as they are able to survive with high loads in their tissue or are able to depurate them (Bryan and Langston, 1992, Smit et al., 2002, Stubblefield et al., 1999, Canli and Atli, 2003, Wallace et al., 2003). These differences in heavy metal sensitivity can result in community shifts in contaminated areas with resistant species replacing more sensitive organisms, often resulting in a lower macroinvertebrate diversity, species richness and evenness (Clements

and Rees, 1997, Warwick, 2001, Solà et al., 2004, Smolders et al., 2003, Mucha et al., 2003, Iwasaki et al., 2009).

Traditional environmental assessments generally consider anthropogenic impacts on biodiversity or community structure but fail to detect more subtle changes in species interactions such as predator-prey relationships (Sánchez-Moreno and Navas, 2007, Jarman et al., 1996). Heavy metals have the potential to affect species interactions due to their tendency to bioaccumulate through the food chain, influence animal behaviour and vary in their toxicity to different species (Rainbow, 2002, Jarman et al., 1996, Draves and Fox, 1998). Furthermore, community shifts at the base of the food web (e.g. from a pollution sensitive community to a more resistant one) can have significant effects on predators at higher trophic levels (Dallinger et al., 1987, Stewart et al., 2004). The assessment of these trophic interactions, however, is complex due to the large amount of small, cryptic or otherwise hard to identify taxa in the diet of many estuarine generalist predators (Symondson, 2002, Asahida et al., 1997). Molecular techniques using DNA extracted from sediment or stomach samples can be used to enhance species identification in environmental and dietary samples with high precision and relatively low effort (see chapter 4), but metabarcoding has not been applied yet to study heavy metal impacts on estuarine communities. The main aim of this chapter is to test the application of trophic metabarcoding of brown shrimp *Crangon crangon* L. stomach contents for environmental assessments in European estuaries. *Crangon crangon* is a suitable test species for environmental and ecotoxicological studies because of its well-known biology, wide geographic distribution, occurrence in contaminated estuaries, economic importance and ease of catch (Menezes et al., 2006, Campos et al., 2009b, Tiews, 1970, Cattrijsse and Makwaia, 1994). Even though *C. crangon* is considered to be sensitive to environmental contamination, it is still able to survive in highly polluted environments (Quintaneiro et al., 2006, Menezes et al., 2009, Dauvin, 2008, Culshaw et al., 2002). By using this crustacean species as a biomarker, I will assess (a) the correlation between heavy metal accumulation in shrimp tissue and environmental pollution levels in six European estuaries; (b) the application of COI-metabarcoding, using nearly universal primers, to describe variation in local benthic community (based on DNA extracted from sediment samples) and *C. crangon* diet (based on DNA extracted from pooled stomach samples) in industrially impacted and reference estuaries (estuaries with a relatively low level of anthropogenic disturbance).

5.3. Methods

5.3.1. Sample collection and processing

Sediment (3 per site) and shrimp (30-50 per site) samples were collected by PVC corer (3.2 mm Ø) and push net from the intertidal zone (0-1m depth) of 22 sites in 6 estuaries with different levels of anthropogenic influences in the United Kingdom (UK), Netherlands and Portugal (Figure 5.1). Estuaries were selected based on the level of industrialisation of their catchment area. The Mersey estuary (UK, 8,914 ha) is a historically contaminated estuary discharging in the Liverpool Bay (Irish Sea) (Irish Sea; Harland et al., 2000, Fox et al., 1999). Its catchment area is highly populated and industrial, encompassing the cities of Liverpool and Manchester. The Kent estuary, on the other hand, is a small estuary (ca. 700 ha) located approximately 100 km to the north of the Mersey estuary with a largely rural catchment area (Halcrow Group Ltd & Kenneth Pye Associates Ltd, 2013). The Kent estuary is connected to the large intertidal area of Morecambe Bay which is a major fishery grounds for *C. crangon* in the UK (Halcrow Group Ltd & Kenneth Pye Associates Ltd, 2013, Henderson et al., 1990). Morecambe Bay can be considered as a reference location for the Liverpool bay area due to its lower pollution levels (Kuncheva et al., 2001, Marshall et al., 2010). In the Netherlands, the Eastern Scheldt (35,000 ha) and Western Scheldt (37,000 ha) are two very similar estuaries in the large river delta of the province of Zeeland. While the Eastern Scheldt is considered an important conservation area and intertidal nursery, the Western Scheldt is highly influenced by the harbour activities and shipping of the upriver city of Antwerp (Hostens and Hamerlynck, 1994, van den Heuvel-Greve, 2009, Jansen et al., 2014, Maulvault et al., 2015, Du Laing et al., 2007). The Minho Estuary (2,300 ha) is located at the border of Spain and Portugal and is considered as a pristine estuary and a suitable reference site for heavy metal studies in Portuguese and European waters (Harland et al., 2000, Mil-Homens et al., 2013, Reis et al., 2008). Ria de Aveiro (43,000 ha) is, on the other hand, located in an urbanised and industrial area and heavy contaminated by industrial effluents (Ramalhosa et al., 2001, Pereira et al., 1998, Martins et al., 2010).

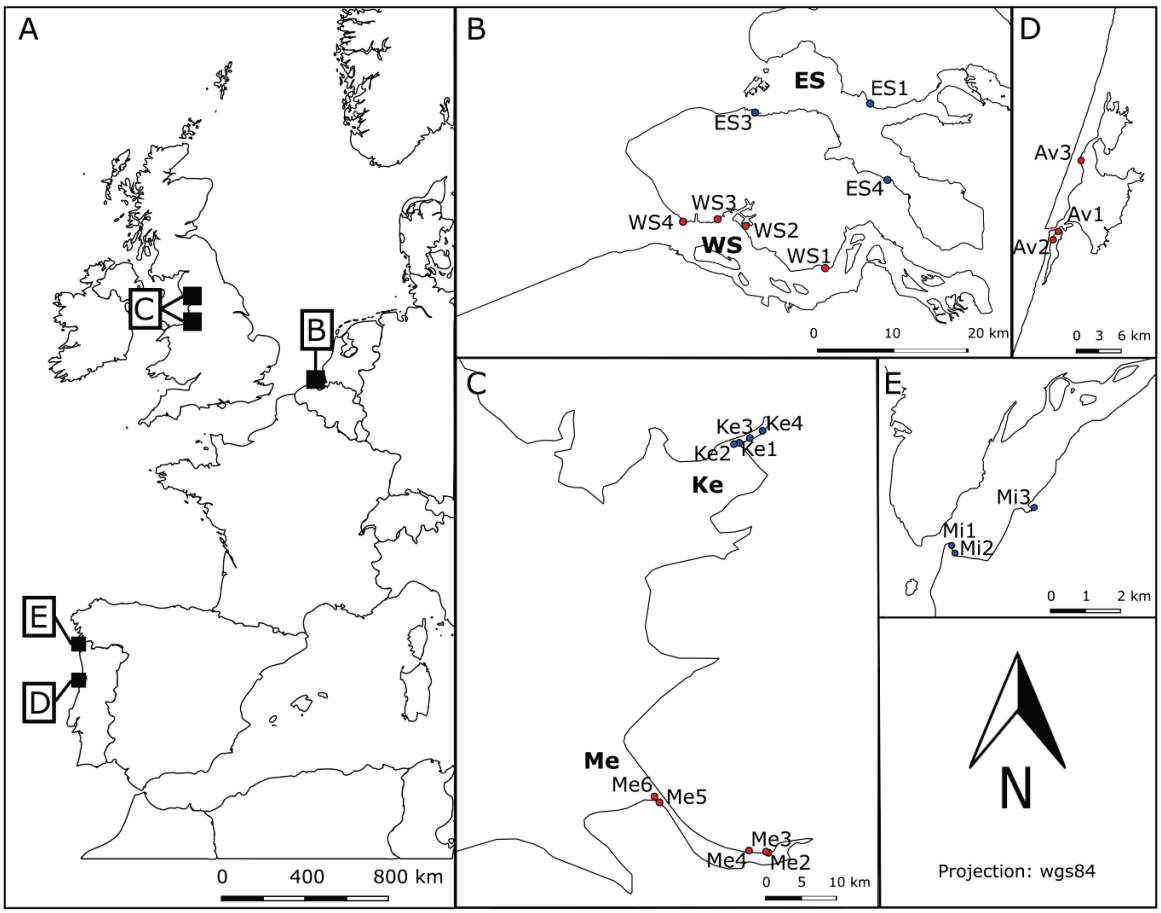

Figure 5.1. Overview of sample locations, illustrating (A) the overall western European scale; (B) the Dutch estuaries, Western Scheldt (WS) and Eastern Scheldt (ES); (C) the British estuaries, Mersey (Me) and Kent (Ke); the Aveiro (D) and Minho (E) estuaries in Portugal. Small dots within estuaries represent individual collection points for shrimp and sediment samples. Red: sites in industrially impacted estuaries; Blue: sites in reference estuaries. Source map: OpenStreetMap.

Caught shrimp were placed on ice and transported to the lab for dissection of their stomach for molecular trophic analysis, and their tail muscle tissue for bioaccumulated heavy metal analyses. Alongside the shrimp required for stomach content analysis, extra shrimp were dissected to complement the muscle tissue samples (see below). Sediment samples were collected in triplicates from the upper 2 cm surface layer at several meters distance from each other. Replicates for heavy metal and granulometric analyses (Horiba LA-950 Particle size analyser) and Total Organic Matter (TOM) determination (by means of ashing: 550°C, 6h) were stored individually in zip-bags while replicates for environmental DNA extraction were pooled per site and stored in 96% ethanol. Water samples were analysed in triplicates for temperature, salinity (Fisher Scientific Traceable Salinity Meter), pH (Hanna HI 98129) and nitrate concentration (Dionex ICS-1000 Ion Chromatography).

5.3.2. Heavy metal determination

Total heavy metal concentrations (de Souza Machado et al., 2016) of sediment (300 mg dry weight (dw)) and pooled shrimp tail muscle tissue samples (250 mg dw; 6-24 shrimp per sample depending on muscle weight) were measured in triplicates per site (in ppm dw). Please note that due to the small size of the shrimp, it was not possible to analyse the same shrimp for trophic content and heavy metal concentration. The muscle tissue of up to 24 shrimps was combined per sample. The bioaccumulated heavy metal concentrations are thus site specific instead of representing individual shrimp levels. Studies on arsenic and cadmium accumulation in *C. crangon* show that although contaminants are accumulated in various tissues, the majority of the accumulation takes place in the tail muscle (Bachmann et al., 1999, Hunter et al., 1998, Niedźwiecka et al., 2011). Samples were air-dried and microwave (Mars Xpress, CEM) digested in nitric acid (65%, Fisher Scientific) for 2 hours prior to analysis by ICP Optical Emission Spectrometry (ICP-OES, Varian 720-ES) using multi-element standards of 0.075, 1 and 10 ppm. The following elements were analysed: Arsenic (As-188), Cadmium (Cd-326), Chromium (Cr-357), Copper (Cu-324), Nickel (Ni-222), Lead (Pb-182) and Zink (Zn-472). Aluminium (Al-167) was also measured for normalization purposes of the sediment metal values (see below; Tweedley et al., 2015). Sediment Cd and tissue Ni concentrations were below the ICP-OES detection limit. Certified reference materials (CRM; Marine sediment: PACS-2, National Research Council Canada; Mussel tissue (*Mytilus edulis*): ERM® - CE278K) and blanks (deionised water) were digested and analysed simultaneously with the samples for quality control (Appendix 5.1).

5.3.3. DNA extraction, high-throughput and bioinformatic analyses

Methods for the DNA extraction, DNA amplification, high-throughput sequencing and bioinformatic analyses of sediment and stomach samples are described in detail in chapter 4. Briefly, sediment and pooled stomach samples (up to 8 stomachs) were extracted using PowerMax® and PowerSoil® DNA isolation Kits (Mo-Bio laboratories). DNA was amplified using a single set of versatile, highly degenerated PCR primers (forward: mICOIintF-XT: 5'-GGWACWRGWTGRACWITITAYCCYCC-3'; reverse: jgHCO2198: 5'-TAIACYTCIGGRTGICCRAARAAYCA-3') targeting the 313-bp COI Leray fragment (Leray et al., 2013; Wangensteen et al., in review, Geller et al., 2013). Amplicons were sequenced on Illumina MiSeq platform using v2 chemistry (2x250 bp paired-ends). Bioinformatic analyses

were performed using the OBITools software suite (Boyer et al., 2016). Amplicon clustering was performed using the SWARM algorithm with a d value of 13 (Mahé et al., 2015, Mahé et al., 2014) and taxonomic assignment was achieved using the ecotag algorithm (Boyer et al., 2016), using a local reference database (Wangensteen et al., in review).

5.3.4. Data analyses

Sequencing reads, heavy metal and environmental data were averaged per site. Blank values were subtracted from the heavy metal results, as per protocol. Concentrations of inert elements such as Al are related to the geology of the sediment and not influenced by anthropogenic sources. The relative proportion of other metals to these inert elements is relatively constant, allowing for the use of heavy metal:AL ratios to assess the degree of anthropogenic enrichment in the sediment (Tweedley et al., 2015, Dauvin, 2008). Sediment heavy metal values were thus aluminium-normalised (calculated as heavy metal:Al ratios) to correct for natural differences in mineralogy and granulometry which are known to influence the contamination level of the sediment (Tweedley et al., 2015, Dauvin, 2008). Data below the detection limit (UD) of the ICP-OES were UD/2 transformed (Ogden, 2010) to allow for the incorporation of these data points in statistical models. Differences between sites in Molecular Taxonomic Unit (MOTU) richness, community structure and relative abundance were studied for the benthic community (based on DNA extracted from sediment samples) in relation to the sediment heavy metal values and environmental terms. Shrimp diet data (based on DNA extracted from pooled stomach samples) were analysed in relation to the site specific bioaccumulated heavy metal concentrations (based on pooled tissue samples) and environmental terms. These analyses were performed for all detected MOTUs and for annelid and arthropod MOTUs specifically. Annelida and Arthropoda are the most important prey groups of *C. crangon* (see chapter 4) and both phyla are known to show variation in sensitivity to heavy metal pollution (Stark, 1998, Neira et al., 2011). Furthermore, factors influencing variations in relative abundances of the potentially endoparasitic fungus *Purpureocillium lilacinum* (chapter 4) were also analysed separately.

All statistical analyses were performed in R v3.1.3 (https://www.R-project.org/) with the vegan (v2.3-5) and lme4 (v1.1-13) packages (Oksanen et al., 2016, Bates et al., 2015). Site and estuary specific correlations in heavy metal and environmental factors were visualised

by means of principal component analyses (PCA). Generalized linear models were constructed (poisson/negative binominal distribution depending on the level of overdispersion) to study the influences of heavy metal and environmental terms on the MOTU richness of the benthic community and *C. crangon* diet. First, MOTU richness was rarefied per sample (to 75% of the lowest read number; Appendix 5.2) to correct for differences in the number of reads using the rrarefy function of Vegan. Second, the median richness of 1000 rarefactions was used as dependent variable in the GLMs. Dependent variables were first tested against all terms individually and a full model was created encompassing all terms with $P < 0.25$. Then, the final model was selected by likelihood ratio tests based on differences in Akaike information criterion (AIC; $P < 0.1$) between the full model and models in which individual terms were removed. The final model was also tested against the Null (intercept) model to determine whether none of the terms were significant (min difference of 2 AIC; Burnham and Anderson, 2002). Differences in community structure between sites were studied based on presence-absence data using canonical correspondence analyses (CCA) and PERMANOVA (Jaccard distances, 1000 permutations) in Vegan. The ordistep function (Vegan) was used to select (both forward and backward selection) the terms for the final CCA and PERMANOVA models. Variation in relative read abundances of annelid, arthropod, and *P. lilacinum* DNA were studied by means of beta regression models (Cribari-Neto and Zeileis, 2010) following similar model selection procedures as described above. Prior to model selection, relative abundances were adjusted to remove any 0 and 1 in accordance to the method of Smithson and Verkuilen (2006)

$$Adjusted\_Score = Original\_Score(N-1)+0.5/N \text{ (N is the total sample size)}$$

5.4. Results

5.4.1. Heavy metal concentrations in sediment and tissue samples

Heavy metal concentrations measured in the sediment varied significantly between estuaries and sites nested within estuaries (Figure 5.2A and Table 5.1). See Appendix 5.3 for the environmental parameters per site. The Mersey estuary was significantly more polluted than most of the other estuaries for all tested elements. Bioaccumulated heavy metal element concentrations in shrimp tissue did not show a significant correlation with concentrations measured in the sediment (raw values and Al- normalized values; Table 5.2), with the exception of Cu raw values (Pearson's correlation; $r_{20} = 0.48$, $P < 0.05$).

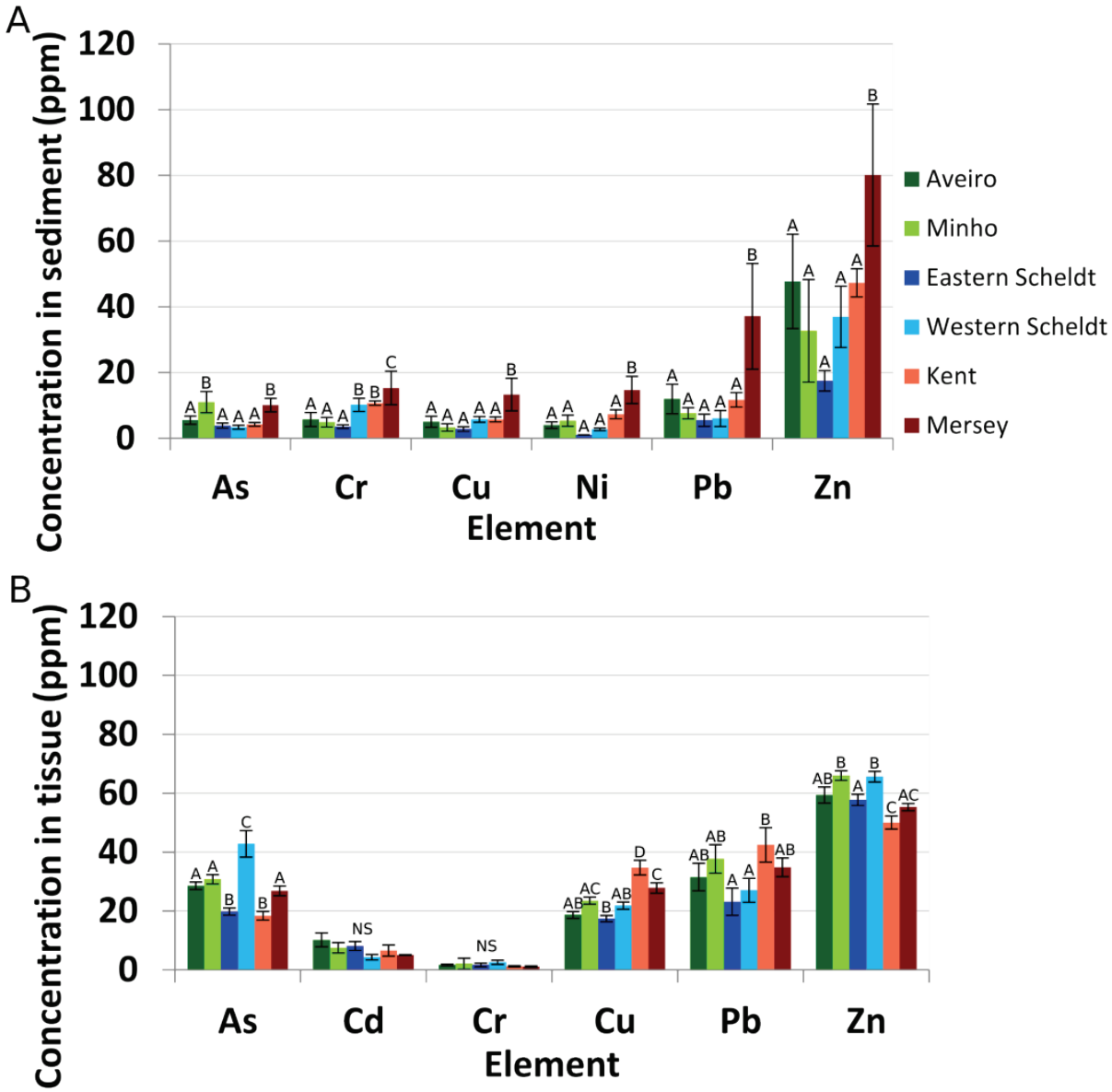

Figure 5.2. Mean (±SE) variation in heavy metal element concentrations (ppm dw) between estuaries measured in (A) sediment samples and (B) *C. crangon* tissue samples. Different letters indicate groups that were significantly different per each element.

Table 5.1. Mean concentrations (ppm dw) and range of elements measured in sediment and *C. crangon* tissue samples and sediment samples collected at different estuaries. Differences between estuaries were tested (one-way ANOVA) between estuaries and sites within estuaries.

		Mean (range) concentration (ppm)						ANOVA					
								Estuary			Site		
		Aveiro	Minho	Eastern Scheldt	Western Scheldt	Kent	Mersey	Df	F	P	Df	F	P
Sediment	As	5.51 (1.31-12.38)	11.00 (5.05-35.05)	3.84 (UD-7.73)	3.36 (UD-6.63)	4.23 (UD-5.93)	10.12 (UD-28.67)	5,37	9.49	<0.001	15,37	8.0	<0.001
	Cd	UD	UD	UD	UD	UD	UD						
	Cr	5.71 (0.83-18.40)	4.88 (1.30-11.98)	3.55 (1.90-6.16)	10.17 (1.37-20.65)	10.76 (8.07-15.16)	15.30 (3.48-72.8)	5,46	24.7	<0.001	17,46	49.2	<0.001
	Cu	5.04 (0.97-13.35)	3.29 (0.52-9.73)	2.81 (UD-5.35)	5.74 (UD-11.81)	5.74 (UD-9.18)	13.39 (1.88-66.62)	5,42	24.0	<0.001	17,42	50.2	<0.001
	Ni	4.00 (1.16-9.48)	5.35 (UD-15.91)	1.04 (UD-1.04)	2.73 (UD-3.16)	7.31 (UD-10.93)	14.74 (UD-40.70)	5,25	18.1	<0.001	9,25	22.3	<0.001
	Pb	11.97 (UD-20.32)	7.60 (UD-12.36)	5.49 (UD-9.84)	6.01 (UD-17.75)	11.75 (UD-20.03)	37.14 (UD-154.03)	5,25	18.9	<0.001	9,25	56.7	<0.001
	Zn	47.73 (3.12-111.28)	32.72 (7.15-151.73)	17:49 (3.30-32.27)	36.96 (8.98-118.8)	47.38 (27.54-70.29)	80.13 (14.62-301.99)	5,46	11.5	<0.001	17,46	18.3	<0.001
Tissue	As	28.56 (23.02-33.35)	30.77 (22.4-35.73)	19.81 (UD-23.75)	42.80 (13.64-56.00)	18.39 (UD-24.32)	26.85 (16.82-35.61)	5,40	43.8	<0.001	15,40	9.7	<0.001
	Cd	10.20 (UD-20.91)	7.51 (UD-13.13)	8.12 (UD -10.71)	4.32 (UD-8.77)	6.58 (UD-12.08)	5.07 (UD-5.20)	5,17	1.5	0.251	5,17	1.0	0.457
	Cr	1.67 (UD-2.11)	2.12 (UD-8.60)	1.67 (UD-4.38)	2.58 (UD-6.36)	1.20 (UD-2.03)	1.11 (UD-3.04)	5,25	1.2	0.321	11,25	2.4	0.035
	Cu	18.67 (13.93-22.98)	23.52 (18.57-29.64)	17.42 (13.42-21.55)	21.82 (18.56-32.17)	34.74 (17.58-52.93)	27.82 (18.59-40.97)	5,42	36.3	<0.001	16,42	6.9	<0.001
	Ni	UD	UD	UD	UD	UD	UD						
	Pb	31.52 (11.33-52.59)	37.70 (21.96-71.65)	23.16 (UD-48.39)	27.02 (11.29-53.11)	42.46 (15.64-68.27)	34.84 (17.01-60.87)	5,42	3.3	0.013	16,42	2.7	0.005
	Zn	59.38 (44.69-69.47)	66.01 (61.2-77.04)	57.74 (49.89-68.51)	65.61 (58.75-76.11)	50.05 (34.42-61.63)	55.28 (49.48-64.43)	5,42	16.1	<0.001	16,42	2.8	0.004

UD: Under limit of detection

Metal concentrations in shrimp tissue varied significantly between estuaries and sites within estuaries for As, Pb and Zn, but not for Cd (Table 5.1). Chromium did not show significant differences between estuaries but varied between sites. Variation in concentrations in shrimp tissue samples among estuaries varied per element and none of the estuaries was significantly more polluted than the others (Figure 5.2B). Principal component analyses (PCA) to show relatedness of the sites based on of the environmental and heavy metal characteristics are displayed in figure 5.3. Total organic matter content and Pb concentration in the sediment were highly correlated (Pearson's correlation; $r_{20} = 0.86$, $P < 0.001$). Tissue Zn concentration was highly correlated with median grain size ($r_{20} = 0.64$, $P < 0.05$), As ($r_{20} = 0.44$, $P < 0.05$) and Cu ($r_{20} = -0.58$, $P < 0.001$) concentrations. Lead (sediment) and Zn (tissue) were therefore removed prior to further analyses while the other variables were kept. There was no clear separation of sites between impacted and reference estuaries in both the PCAs based on the metal concentrations in sediment (Al-normalised) and shrimp tissue (Figure 5.3).

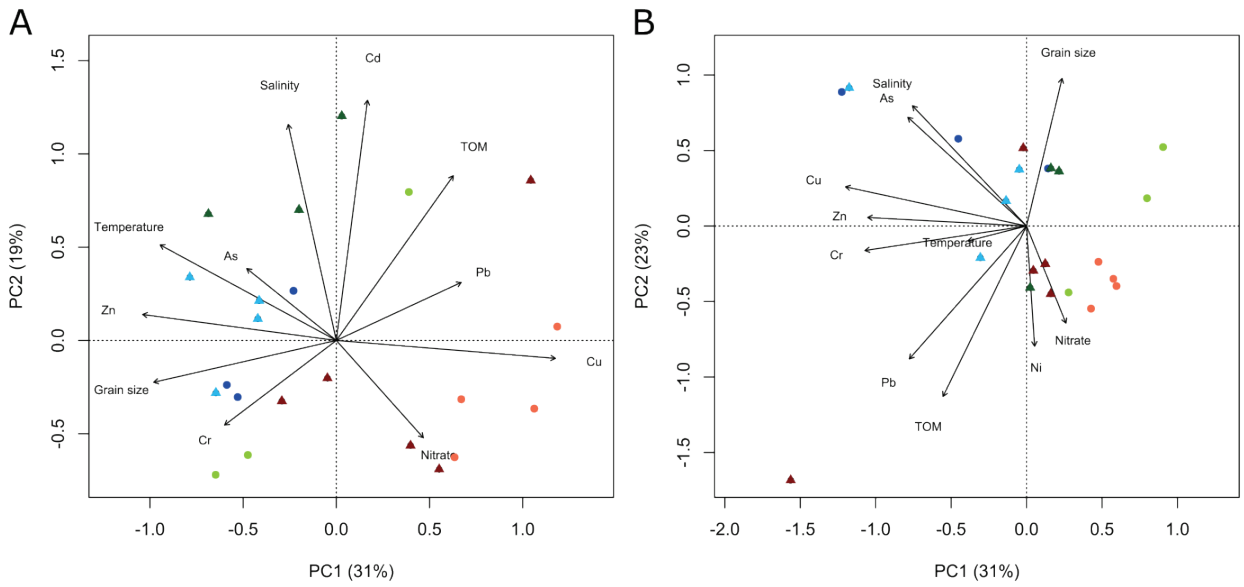

Figure 5.3. Principle component analysis scores and vectors for environmental variables and heavy metal element concentrations measured in (A) sediment samples (Al-normalised) and (B) *Crangon crangon* tissue samples. Dots represent reference sites and triangles indicate industrial impacted sites. Estuaries are identified by colour; dark green = Aveiro; light green = Minho; light blue = Western Scheldt; dark blue = Eastern Scheldt; dark red = Mersey; light red = Kent.

Table 5.2. Pearson's correlations of heavy metals measured in *C. crangon* tissue samples versus heavy metals measured in sediment samples (raw values and Aluminium-normalised). ***** P < 0.05

	Tissue vs. sediment					Tissue vs. Al-normalized sediment				
	As	**Cr**	**Cu**	**Pb**	**Zn**	**As**	**Cr**	**Cu**	**Pb**	**Zn**
Df	20	20	20	20	20	20	20	20	20	20
r	0.07	0.03	0.48	0.29	-0.27	0.17	0.27	-0.06	0.21	0.12
P	0.754	0.903	0.02*	0.187	0.224	0.45	0.229	0.775	0.342	0.607

5.4.2. MOTU richness

5.4.2.1. Benthic community from sediment samples

Benthic total MOTU and arthropod and annelid richness in the sediment samples were not correlated to Al-transformed heavy metal concentrations in the sediment, but there were significant differences in total MOTU richness between estuaries, while this was not the case for annelid and arthropod richness (Table 5.3).

5.4.2.2. Shrimp diet

Bioaccumulated heavy metal concentrations in *C. crangon* tissue samples showed several significant relationships with rarefied MOTU richness in the shrimp stomach samples, depending on the element and taxonomic group tested (Table 5.3). Total MOTU richness of taxa detected in shrimp stomach samples was significantly higher at sites with high As concentrations in shrimp tissue (coefficient: 0.34, P = 0.007) but lower at sites with high Cu bioaccumulation (coefficient: -0.31, P = 0.03). Also annelid richness showed a weak negative relationship with sites with high Cu bioaccumulation (coefficient: -0.06, P = 0.02). On the other hand, arthropod richness was higher in stomachs collected from sites with higher As (coefficient: 0.04, P = 0,004), Cr (coefficient: 0.30, P = 0.003), Cu (coefficient: 0.05, P = 0.03) and Cd (coefficient: 0.16, P < 0.001) concentrations in shrimp tissue, but the effect size varied substantially per element. Arthropod richness also showed a weak negative correlation with salinity and positive correlation with median grain size (Table 5.3). MOTU richness (total, annelid and arthropod) in the stomach samples was not influenced by the number of stomachs pooled per site but differed significantly between estuaries (LRT: P < 0.05; Table 5.3).

Table 5.3. General linear model coefficient estimates describing the relationship between rarefied MOTU richness of different taxonomic groups detected in DNA extracted from either sediment or pooled *Crangon crangon* stomach samples and environmental and heavy metal element concentrations (ppm dw). Heavy metal elements were measured in either sediment samples or *C. crangon* muscle tissue samples. Sediment heavy metal levels were Aluminium-normalised prior to analysis. Only the results of the final models (selected based on likelihood ratio tests (LRT) of the individual terms) are shown. The factor Estuary was included/excluded in the final models based on LRT ($P < 0.05$). Terms not included in the final models are represented by empty cells. Empty lines indicate that the final model AIC was not significantly lower from the null (intercept) model AIC, based on LRT. See appendix 5.2 for the number of reads each taxonomic group was rarefied to.

DNA	HM	MOTUs tested	Temp. (°C)	Salinity	Grain Size	NO_{3-}	As	Cr	Cu	Pb	Cd	Stomachs (N)	Estuary[3]
Sediment	Sediment	All MOTU[1]										NA	Yes
		Annelida[2]										NA	
		Arthropoda[2]										NA	
Stomach	Tissue	All MOTU[1]					0.34*		-0.31*				Yes
		Annelida[2]							-0.06*			0.05	Yes
		Arthropoda[2]		-0.09*	0.01*		0.04*	0.3*	0.05*		0.16*		Yes

HM: Heavy metal source; 1: Negative binomial distribution; 2: Poisson distribution; 3: Based on LRT; *: $P < 0.05$

5.4.3. Multivariate analyses on community structure

5.4.3.1. Benthic community from sediment samples

Model selection of CCA-models using ordistep resulted in the inclusion of different terms in the final benthic community CCA and PERMANOVA models (Table 5.4). Al-normalised Cr, Cu and Ni concentrations measured in the sediment were included in the final CCA-model considering all MOTUs, but PERMANOVA analyses showed that all these terms were not significant (Figure 5.4; Cr: pseudo-F: 1.15, P = 0.171; Cu: pseudo-F: 1.22, P = 0.099; Ni: pseudo-F: 1.03, P = 0.369). Variation in community structure in the sediment samples was mainly related to differences between estuaries (Table 5.4).

5.4.3.2. Shrimp diet

Model selection included either salinity and median grain size or estuary as significant explanatory variables to the final multivariate models on MOTU composition in *C. crangon* stomach samples (Table 5.4). Annelid MOTU composition varied with bioaccumulated As, Cr and Pb concentrations in *C. crangon* tissue, but these terms were not significant (Figure 5.5; PERMANOVA: As: pseudo-F: 1.16, P = 0.225; Cr: pseudo-F: 1.17, P = 0.232; Pb: pseudo-F: 1.23, P = 0.163).

Table 5.4. Terms included in Canonical correspondence analysis (CCA) of presence-absence data on different (sub)sets of MOTUs detected in sediment and *Crangon crangon* pooled stomach samples. Heavy metal concentrations were determined from either sediment or *C. crangon* tissue samples. Model selection was performed using the Ordistep function of Vegan. P-values (based on PERMANOVA analyses) are given for factors included in the CCA-models. Terms not included in the final models are represented by empty cells. HM: source of heavy metal elements (sediment (aluminium-normalised) or *C. crangon* tissue).

DNA	HM	Dataset	Temp. (°C)	Salinity	Grain Size	NO_3-	As	Cr	Cu	Ni	Pb	Estuary	Var.[1]
Sediment	Sediment	All MOTUs		0.064	0.198	0.094		0.171	0.099	0.369		0.001*	60%
		Annelida										0.006*	37%
		Arthropoda		0.075		0.289						0.001*	39%
Stomach	Tissue	All MOTUs										0.032*	26%
		Annelida		0.063			0.225	0.232			0.163	0.025*	54%
		Arthropoda		0.020*	0.001*								15%

* = P < 0.05. 1: Variance (Var.; %) explained by the constrained CCA axes

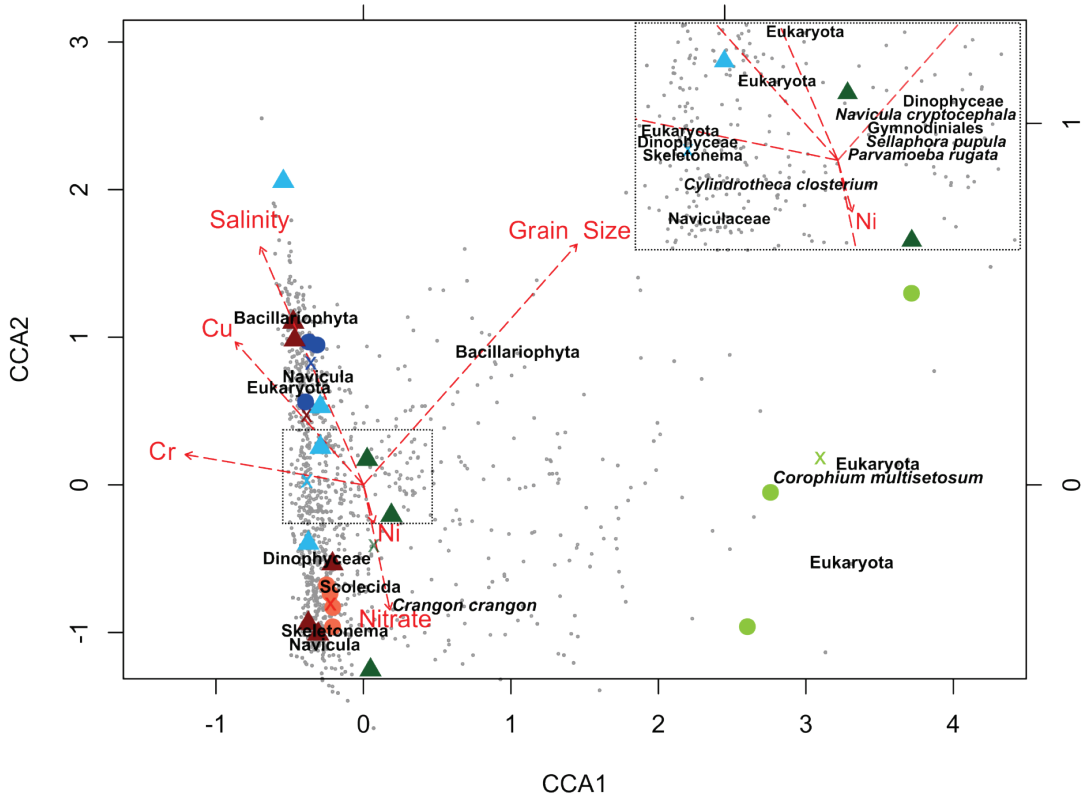

Figure 5.4. Canonical correspondence analysis (CCA) of presence-absence data of MOTUs detected in sediment samples collected in 6 estuaries. Copper (Cu) and Chromium (Cr) concentrations were measured in sediment samples and aluminium-normalised. Grey dots represent the MOTU scores and taxa names are provided for abundant MOTUs (≥0.5% read abundance). The inset zooms in on the centre of the graph. Site scores are indicated with large dots (reference sites) or triangles (industrial impacted sites). PERMANOVA (Table 5.5) analysis showed that, with the exception of the factor estuary (scores are indicated by crosses), none of other terms (dashed lines) had a significant influence on the MOTU scores. Estuaries: Dark green = Aveiro; light green = Minho; light blue = Western Scheldt; dark blue = Eastern Scheldt; dark red = Mersey; light red = Kent.

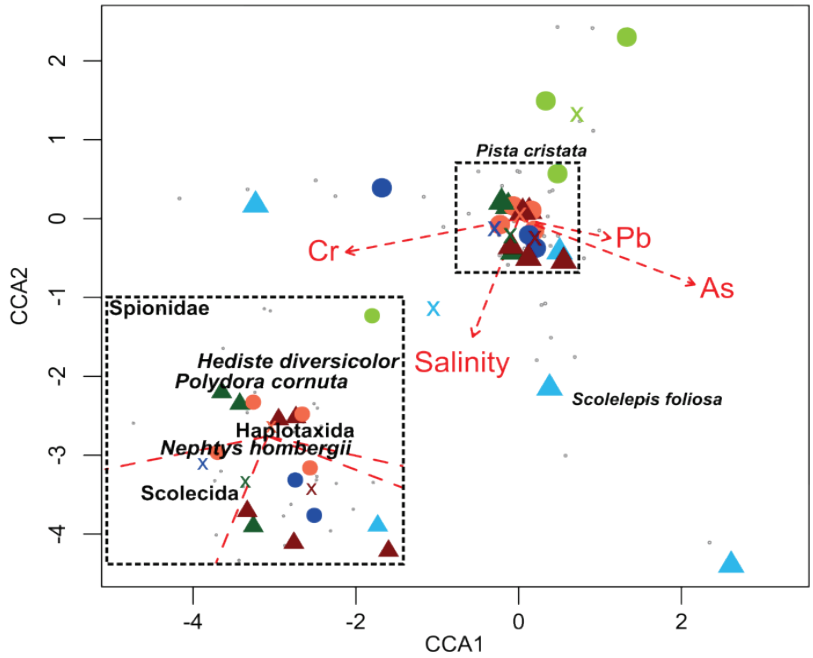

Figure 5.5. Canonical correspondence analysis (CCA) of presence-absence data of annelid MOTUs detected in pooled *Crangon crangon* stomach samples in relation to salinity, median grain size and bioaccumulated heavy metal elements. Grey dots represent the MOTU scores and taxa names are provided for abundant MOTUs (≥ 0.01% read abundance). The insets zoom in on the centres of the graphs. Site scores are indicated with large dots (reference sites) or triangles (industrial impacted sites). PERMANOVA (Table 5.5) analysis showed that, with the exception of the factor estuary (scores are indicated by crosses), none of other terms (dashed lines) had a significant influence on the MOTU scores. Estuaries: dark green = Aveiro; light green = Minho; light blue = Western Scheldt; dark blue = Eastern Scheldt; dark red = Mersey; light red = Kent.

5.4.4. Relative abundances

5.4.4.1. Benthic community from sediment samples

Betamodel selection (based on LRT) did not reveal significant relationships between the relative abundance of *P. lilacinum* and arthropod DNA detected in the sediment samples and environmental or heavy metal terms (Table 5.6). Annelid relative abundance was also not related to sediment heavy metal concentrations (Al-transformed), but showed a significant positive relationship with water temperature (Table 5.6).

5.4.4.2. Shrimp diet

Purpureocillium lilacinum was significantly more abundant in stomach samples collected at sites with higher bioaccumulated Cu levels in shrimp tissue and varied significantly between estuaries (Table 5.6). Relative arthropod and annelid read abundances in the stomach samples did not vary significantly with any of the terms tested (Table 5.6).

Table 5.6. Coefficient estimates and probabilities of the effects of environmental terms and heavy metal concentrations (measured in *Crangon crangon* tissue or sediment samples) on the relative read abundances of the fungus *Purpureocillium lilacinum*, arthropods and annelids detected in sediment and *C. crangon* pooled stomach samples. Heavy metal levels in the sediment were Al-transformed prior to analyses. Only terms included in the final betamodels are shown.

Taxa	DNA substrate	Metal substrate	Term	Coefficient ± SE	Z/Chisq[2]	P
P. lilacinum	Sediment	Sediment[1]	NA			
	Stomach	Tissue	Cu	0.58 ± 0.28	2.1	**0.040***
			Estuary		14.8[2]	**0.011***
Arthropoda	Sediment	Sediment[1]	NA			
	Stomach	Tissue	NA			
Annelida	Sediment	Sediment[1]	Temperature	0.30 ± 0.1	3.9	**<0.001***
	Stomach	Tissue	NA			

NA: AIC final model was not significantly lower than from the intercept model; 1: Metal element concentrations are Al-normalised; 2: Based on LRT. * $P < 0.05$

5.5. Discussion

Environmental impact assessments require precise and reliable estimates of biodiversity and community structure. Monitoring of anthropogenic impacts in key ecosystems such as estuaries is essential due to the ecological and economical importance of these systems (Martínez et al., 2007, Sheaves et al., 2015). Recent studies include molecular tools alongside traditional methods to assess variation in species diversity and community structure in a range of aquatic and marine habitats (Shaw et al., 2016, Valentini et al., 2016, Guardiola et al., 2015). Alongside these important indicators of ecosystem health, the study of trophic relationships is also essential for monitoring anthropogenic impacts (Stewart et al., 2004, Hogsden and Harding, 2012). This study assessed the bioaccumulation of heavy metals in the brown shrimp and applied metabarcoding to detect variation in benthic community structure and trophic ecology of a crustacean key species in European coastal waters.

5.5.1. Heavy metal accumulation in sediment and tissue samples

A thorough understanding of the accumulation of pollutants from environment into animal tissue is essential for the assessment of anthropogenic impacts. Alongside contaminant measurements in water and sediment, many studies focus on accumulated contaminant levels in biota since they more relevant from an ecological and human health perspective (in case of edible species; Van Ael et al., 2017, Conti et al., 2017, Jayaprakash et al., 2015, Rainbow, 2002). Conversely, heavy metal concentrations in the sediment and biota do generally not correlate well due to low mobilisation rates of metals in the sediment (Phillips, 1977, Rainbow, 2009). Furthermore, heavy metal accumulation rates can differ between and within species depending on environmental and physiological conditions of the animal (Neira et al., 2011, Van Ael et al., 2017, Engel and Brouwer, 1987, Hunter et al., 1998). This low level of correlation between bioaccumulated and sediment heavy metal levels was also clear during the present study since Cu was the only element that showed a significant correlation. Interestingly, Cu is an essential metal which generally does not follow environmental concentrations since it is regulated by the animal and varies in accordance to physiological processes, such as its intermoult cycle (Bachmann et al., 1999, Hunter et al., 1998, Niedźwiecka et al., 2011, Andersen et al., 1984, Szaniawska, 1985). The motile and migratory behaviour of the study organism also needs to be taken into consideration. The brown shrimp is highly mobile, especially during the night, and shows seasonal migrations

(Lloyd and Yonge, 1947, Donk and Wilde, 1981). Thus, bioaccumulated heavy metal levels of motile species such as *C. crangon* might not be the most suitable proxies for site specific pollution compared to more sessile species such as mussels, oysters and barnacles (Rainbow, 1995).

Estuary wide heavy metal concentration patterns in the sediment did not vary in accordance to the classification of "impacted" and "reference" estuaries. The Mersey estuary was significantly more polluted than the other estuaries, but other impacted estuaries did not differ in pollution levels from their neighbouring reference estuaries. Extensive environmental remediation efforts conducted in European rivers and estuaries during the last decade might have contributed to the reduction of the expected variation in pollutant concentrations (Vink et al., 1999, Markmann and Tautz, 2005). Even the Mersey estuary, which has a history of pollution since the industrial revolution, has seen a major increase of biological quality, resulting in the return of fishes (Jones, 2000, Jones, 2006). Heavy metal concentrations detected during the present study were generally low (Neira et al., 2011, Stark, 1998) and, therefore, difficult to detect accurately by ICP-OES (as shown by the high error of the Cd and Pb CRM levels; Appendix 5.1), which might also have contributed to the lack of differences detected between estuaries.

The absence of a clear difference in pollution levels between industrial and reference estuaries was reflected by the lack of separation of these estuaries in the multivariate analyses based on the benthic community samples and shrimp diet. Alongside the possible effects of the remediation efforts discussed above, anthropogenic impacts on communities living in naturally stressed environments are generally difficult to detect since these communities are well adapted to high levels of disturbance (Elliott and Quintino, 2007). Estuaries show also large temporal and spatial variation in abiotic factors, such as salinity and grain size, which are known to influence both local species composition and heavy metal accumulation and toxicity (Bryan and Langston, 1992, Mayer-Pinto et al., 2010). These confounding factors, together with the generally patch distribution of heavy metal levels in sediments, complicate conclusions drawn at larger spatial scales and call for approaches considering element gradients on a site-by-site basis instead of estuary wide assessments (Li et al., 2017, Coynel et al., 2016, Chapman and Wang, 2001), as discussed below.

5.5.2. Benthic community responses to heavy metal pollution

Variation in benthic community structure was not related to heavy metal levels measured in the sediment. Heavy metals might influence aquatic communities by reduction of overall diversity, changes in the abundance of specific species or by indirect ecological effects (Mayer-Pinto et al., 2010). These influences, however, are generally of secondary importance to the effects of gradients of natural abiotic factors, and difficult to detect in complex systems (Oug et al., 2012, Sanchez-Moyano and Garcia-Asencio, 2010, Chariton et al., 2015, Dauvin, 2008). During the present study, benthic MOTU richness and community structure showed significant differences between estuaries, but no environmental terms were detected that might explain these differences.

5.5.3. Variation in *Crangon crangon* diet in relation to heavy metal pollution

Prey consumption and bioaccumulation of heavy metals are interlinked due to the importance of diet as a pathway of metal accumulation (Hunter et al., 1998, Niedźwiecka et al., 2011, Bachmann et al., 1999). Predators consuming prey with high metal loading will thus likely show higher metal accumulation (Sánchez-Moreno and Navas, 2007, Jarman et al., 1996). On the other hand, data on bioaccumulated heavy metals, obtained either through food, water or sediment, can provide information on environmental conditions of the predator's habitat and the prey species that might be encountered in those habitats (Clements and Rees, 1997, Hogsden and Harding, 2012). To complicate this relationship further, several heavy metals can influence the senses and behaviour of both preys and predators and could thus potentially influence the food selection (Nilsson Sköld et al., 2013, Blinova and Cherkashin, 2012, Simbeya et al., 2012, Mills et al., 2006, Carreau and Pyle, 2005). Although it is consequentially not possible to draw any causal relationships based on the data obtained during this study, the approach used should be employed more often as it can contribute to a more comprehensive understanding of this complex system and be of value for the development of manipulative experiments which are required to confirm the observed relationships between predator diet and internal/external heavy metal concentrations.

In the first place, the results show that the influence of heavy metal contaminants on the shrimp's diet is likely of secondary importance after variation in natural factors such as

salinity and grainsize (in line with chapter 4). As discussed with regard to heavy metal impacts on benthic communities, variation in natural stressors as salinity often have a more pronounced impact on benthic communities than pollutants (Oug et al., 2012, Sanchez-Moyano and Garcia-Asencio, 2010, Chariton et al., 2015, Dauvin, 2008). Since *C. crangon*'s opportunistic diet reflects the local benthic community (Chapter 4; Oh et al., 2001, Pihl, 1985), these natural factors will also be of influence on its diet. Nonetheless, in contrast to the lack of heavy metal effects detected on the benthic community structure, the concentrations of some bioaccumulated heavy metals were correlated with variation in the brown shrimp's diet. For example, shrimp caught at sites with high levels of bioaccumulated Cu had a less diverse diet.

Copper is generally considered to be one of the more toxic heavy metals to invertebrates (Stark, 1998, Neira et al., 2011, Reish, 1993). Continuous exposure to Cu could result in a variety of detrimental effects including behavioural impairments, lower survival and reduced reproduction (Bielmyer et al., 2006, Mirza and Pyle, 2009). Copper toxicity varies between species (Neira et al., 2011, Sanchez-Moyano and Garcia-Asencio, 2010, Rygg, 1985) and the observed slight increase in the arthropod MOTU richness found in the shrimp stomachs at sites with higher levels of bioaccumulated Cu might have been, partly, related to prey-specific sensitivities to local Cu concentrations. Several other heavy metals (As, Cr and Cd) also showed a positive relationship with arthropod richness detected in stomachs. The overall MOTU richness in the shrimp's diet was, furthermore, higher at site with high levels of As bioaccumulation. These positive relationships might be partly explained by the relatively low toxicity of some of these elements, especially in the case of As where the chemical species found in marine biota are generally less toxic (Eisler and Hennekey, 1977, Dauvin, 2008, Francesconi and Edmonds, 1998). But, many other variables might have also contributed to these positive relationships (and the negative ones described above) since contamination studies in estuaries are prone to many potentially confounding factors and pollution might result in indirect ecological interactions due to the complex relationships between the species (e.g. competition and predation; Chapman and Wang, 2001, Mayer-Pinto et al., 2010). Given the complexity of this topic, the question might be asked whether shrimp tissue metal concentrations are the best proxy for local pollution.

Second, this study provided insights in the possible links between ecotoxicology and endoparasites or symbionts. The fungus *P. lilacinum* is highly present in the digestive system

of *C. crangon* (see chapter 4), probably acting as an endoparasite (Castillo Lopez et al., 2014, Nidheesh et al., 2015). Here, a possible relationship between *P. lilacinum* abundance in shrimp stomachs and heavy metal concentrations was detected. The abundance of this fungus in *C. crangon* stomachs was positively related to bioaccumulated Cu concentrations. On the other hand, no relationship between sediment dwelling *P. lilacinum* abundance and heavy metal contamination in the sediment was detected. *Purpureocillium lilacinum* survives well in contaminated habitats and has been isolated in areas with elevated levels of heavy metals, including Cd, Zn, Cu and Pb (Oggerin et al., 2013, Zeng et al., 2013). It has also been shown that this species can promote the growth of mangrove plants under copper stress, possibly by acidifying the soil, reducing the toxicity of Cu (Gong et al., 2017). The precise relationship between *P. lilacinum* and *C. crangon* has not been investigated yet (see chapter 4) and correlations between shrimp heavy metal loadings and *P. lilacinum* growth in their digestive system can have multiple explanations, ranging from increase rates of parasitism to commensalistic/mutualistic interactions. A comprehensive understanding of endoparasitic and symbiotic interactions is essential for assessing trophic relationships and crustacean diseases (Coors and De Meester, 2008, Dyrynda, 1998), and this study could act as a basis for future studies on the link between crustacean endoparasitology and ecotoxicology.

5.5.4. Conclusions and recommendations

This study provides important insights on the anthropogenic processes that play a role in structuring benthic communities in European estuaries, and investigated links between the trophic ecology and ecotoxicology of a widely distributed generalist predator. The importance of estuaries as nursery grounds for many species and the ecosystem services provided by these systems makes the monitoring of anthropogenic impacts in these habitats essential (Sheaves et al., 2015, Martínez et al., 2007, Mayer-Pinto et al., 2010). Determination of species diversity and richness of benthic and infaunal communities is, however, often complicated since many species are hard to identify within these communities (Hajibabaei et al., 2011, Lejzerowicz et al., 2015). As a result, there is often a discrepancy in the precision of the biological (often detected at higher taxonomic ranks only) and toxicological data (often represented in ppm or ppb) in environmental assessments (Chariton et al., 2014, Joachim et al., 2017, Sánchez-Moreno and Navas, 2007). Low taxonomic precision can result in errors in the assessment of anthropogenic impacts or the

determination of indicator species since closely related species may have completely different responses and sensitivities to contaminants (Neira et al., 2011, Frank and Robertson, 1979, Reish, 1993). The higher taxonomic precision that can be gained by the application of molecular tools in environmental monitoring is, therefore, a substantial improvement compared to traditional identification methods. These molecular tools allow for rapid and extensive assessments of multiple taxa without the requirement of extensive taxonomic skills (Goodwin et al., 2017, Chariton et al., 2014, Chariton et al., 2015, Pawlowski et al., 2014).

Several field and mesocosm studies have already successfully applied molecular skills to monitor variation in fish, macroinvertebrate and benthic diversity in relation to natural and anthropogenic pressures (Hajibabaei et al., 2011, Evans and Lamberti, 2017, Chariton et al., 2014, Pawlowski et al., 2014). Molecular trophic data has not yet been incorporated in these studies, while the importance of incorporating species interactions in environmental impact assessments is well recognised (Sánchez-Moreno and Navas, 2007, Wang, 2002, Joachim et al., 2017). The incorporation of species networks in ecotoxicological studies can reveal essential links between prey consumption and pollution impacts including prey-specific metal accumulation, reduction of food intake and growth rates, changes in prey abundance, and the use of diet subsidies (replacing disappearing prey species with other food items; Stewart et al., 2004, Kraus et al., 2016, Kövecses et al., 2005). Food is an important pathway of pollutant accumulation and some heavy metals can be biomagnified in higher trophic levels (Wang, 2002, Hunter et al., 1998, Jarman et al., 1996). The integration of molecular trophic information in ecotoxicological studies is, therefore, essential to reveal anthropogenic impacts on species interactions which would not be detected with traditional biodiversity assessments. This study is, to the author's knowledge, the first study applying trophic molecular tools to study the impacts of heavy metal pollution in estuarine systems and could provide the basis of future manipulative experiments either in the field or the laboratory. Manipulative experiments could either be conducted in a mesocosm system or along a pollution gradient in a semi-enclosed system. A controlled environment is required to reduce the influence of the many confounding factors which play a major role in estuarine systems and limit the ability of observational studies, such as the present one, to demonstrate causal relationships (Chapman and Wang, 2001, Mayer-Pinto et al., 2010, Wright, 1995). The detected correlations between the diet of an opportunistic predator and

several heavy metal elements show that the application of metabarcoding and the inclusion of trophic information can be a valuable addition to traditional environmental impact assessments in order to gain comprehensive insights in the responses of estuarine communities to anthropogenic stressors, including the effects on predator-prey and parasitic interactions.

Chapter 6.

Metabarcoding of shrimp stomach content: harnessing a natural sampler for fish biodiversity monitoring

6.1. Abstract

Given their positioning and biological productivity, estuaries have long represented key providers of ecosystem services, and consequently remain under remarkable pressure from numerous forms of anthropogenic impact. The monitoring of fish communities in space and time are one of the most widespread and established approaches to assess the ecological status of estuaries and other coastal habitats, but traditional fish surveys are invasive, costly, labour intensive and highly selective. Recently, the application of metabarcoding techniques, on either sediment or aqueous environmental DNA, has rapidly gained popularity. Here, we evaluate the application of a novel, high through-put DNA-based monitoring tool to assess fish diversity, based on the analysis of the gut contents of a generalist predator/scavenger, the European brown shrimp, *Crangon crangon*. Sediment and shrimp samples were collected from eight European estuaries and DNA metabarcoding (using both 12S and COI markers) was carried out to infer fish assemblage composition. We detected 32 teleost species (16 and 20, for 12S and COI respectively). Twice as many species were recovered using metabarcoding than by traditional net surveys. A combination of multiple markers and sample types (stomach and sediment) revealed a comprehensive picture of European estuarine fish diversity, with no significant large-scale geographical differentiation among estuaries. By comparing and interweaving trophic, environmental DNA and traditional survey-based techniques, we show that the DNA-assisted gut content analysis of a ubiquitous, easily accessible, generalist species may serve as a powerful, rapid and cost-effective tool for large scale, routine estuarine biodiversity monitoring.

6.2. Introduction

Accurate and reliable estimates of biodiversity and species distributions are essential for successful ecosystem management and environmental policy (Hooper et al., 2005, Rees et al., 2014). Understanding biodiversity changes in coastal systems, such as estuaries, is of special interest since these provide essential ecosystem functions and services and are heavily affected by anthropogenic pressures (Halpern et al., 2008, Worm et al., 2006, Sheaves et al., 2015). Estuaries are highly productive systems, providing food and shelter for a large range of fish and invertebrates (Beck et al., 2001, Heip et al., 1995). These habitats act as important nurseries for many fish species, resulting in a greater density, survival rate and growth of juveniles than surrounding habitats (Beck et al., 2001, Kraus and Secor, 2005), which explains the adaptations and energy required for fish larvae to migrate from the open sea to estuaries (Huijbers et al., 2012, Norcross and Shaw, 1984). Alongside their importance as nursery areas, estuaries also support a wide range of adult fish species including estuarine residents, marine and fresh water "stragglers" (taxa normally occurring in marine habitats), and migratory species (Elliott and Dewailly, 1995, Elliott et al., 2007). Many of these are important targets for fisheries or key-stone elements for coastal food webs and of relevance for global economy and food security (Jovanovic et al., 2007, Wilson, 2002, Pauly et al., 2005, Scheffer et al., 2005). A thorough understanding of the community structure, spatial distribution, population connectivity and prey selection of bony fish is essential for ecosystem characterisation and management (Mariani et al., 2011, Jovanovic et al., 2007, Kraus and Secor, 2005, Genner et al., 2004). This is becoming crucial since ichthyofaunal communities are under pressure of a range of anthropogenic impacts such as overfishing, pollution and climate change (Genner et al., 2004, Wilson, 2002, Courrat et al., 2009). Due to these pressures, fish communities are generally considered to be suitable biological indicators for the environmental quality of estuarine systems, as monitoring fish communities integrates the direct and indirect effects of stressors on the entire aquatic ecosystem (Whitfield, 2002, Fausch et al., 1990). Fish surveys are regularly conducted for the management of oceanic and transitional waters, fisheries stock assessments, detection of invasive species, monitoring of environmental changes, water quality assessments, etc. (Pyšek and Richardson, 2010), and are required to comply with environmental policy such as the EU Water Framework Directive for Transitional Waters (Ferreira et al., 2007).

Traditional estimates of fish diversity largely depend on fish captures which are usually invasive, costly, labour intensive and selective (Lapointe et al., 2011, Cotter et al., 2004, Thomsen et al., 2012). Recent molecular biodiversity assessment methods, such as environmental metabarcoding, focus on detecting animals' presence by collecting the DNA they have left behind in the environment (Taberlet et al., 2012a, Thomsen et al., 2012) and applying high-throughput sequencing (HTS) to identify multiple taxa based on bulk DNA extracted from a community (DNA derived from many individual organisms, representing several species) or environmental sample (i.e. water, soil, faeces; Barnes and Turner, 2016). Metabarcoding can successfully identify small, cryptic or decomposed specimens with reduced cost and effort compared to traditional methods, and is independent of the species' developmental stage (Chariton et al., 2015, Leray and Knowlton, 2015, Hajibabaei et al., 2011, Lejzerowicz et al., 2015), though this may also represent a limitation, when that type of information is required (Valentini et al., 2016).

A recent metabarcoding development is the use of DNA detected in the gut contents of parasitic/predatory organisms to estimate the diversity and distribution of their prey items. Molecular trophic tools have advantages over traditional taxonomic methods since the stomachs of animals often contain a high proportion of material that is very difficult to identify with traditional microscopic identification, such as small, soft bodied, and highly digested prey (Symondson, 2002). The application of leeches and carrion flies as biodiversity sampling tools has been proposed for the rapid assessment of mammals in several terrestrial habitats (Calvignac-Spencer et al., 2013b, Schnell et al., 2015b, Schnell et al., 2012). Although the concept of examining species distribution based on their detection as prey items in the stomach contents of predators has been applied using traditional morphological methods (e.g. Stevens et al., 2010, Lasley-Rasher et al., 2015, Boucek and Rehage, 2014, Fahrig et al., 1993), trophic DNA-based methods for biodiversity assessment have not yet been employed in marine systems, and much still needs to be done in order to identify the most appropriate sample types and markers to detect specific biodiversity components, such as, for instance, teleost species (Shaw et al., 2016).

Here, I focus on the applicability of metabarcoding of DNA extracted from the stomach contents of an opportunistic scavenger/predator, the brown shrimp, *Crangon crangon* L., as a sampling tool for fish diversity in European coastal waters. The brown shrimp is a widespread and abundant decapod crustacean in European soft bottom habitats (Campos

and van der Veer, 2008, Bamber and Henderson, 1994). Its opportunistic diet makes it a very suitable candidate as a biodiversity sampling tool (Oh et al., 2001, Pihl and Rosenberg, 1984) and a recent study shows that COI metabarcoding of its stomach contents can reveal a wide range of prey items including multiple fish taxa (Chapter 4). Besides the main general objective, to evaluate the suitability of shrimp stomach content to assess fish diversity, three secondary objectives are evaluated, namely i) to test the effectiveness of the proposed method to assess regional variations in fish diversity, ii) to compare the efficacy of different DNA sample media to detect fish taxa, and iii) to compare fish communities identified via metabarcoding surveys with those identified from concurrent net surveys.

6.3. Methods

This study comprised two independent and complimentary efforts. First, DNA extracts from *C. crangon* stomach and sediment samples collected from 6 European estuaries were amplified with two different markers (COI and 12S) and the detected fish taxa were evaluated and compared in relation to sample type, marker and location. Secondly we focused on two British estuaries to compare the detection of fish environmental DNA (envDNA) extracted from shrimp stomachs, water and sediment substrates, with morphological identification of fish caught in concurrent seine net surveys.

6.3.1. Sample collection and processing

To evaluate the suitability of trophic contents to assess fish diversity, adult brown shrimp (20-50 mm total length) and sediment samples were collected from the intertidal zone (0-1m depth) at 21 sites distributed over 6 estuaries in the Netherlands, Portugal and the United Kingdom (Figure 6.1), between May and July 2016. Shrimp were collected by push-net at low tide (±3h). Sediment samples were collected with a PVC corer (3.2 mm Ø) from the upper 2 cm surface layer (which represent the most recent DNA deposits; Turner et al., 2015, Limburg and Weider, 2002) and 3 subsamples were pooled per site to reduce the influence of local heterogeneity (Taberlet et al., 2012b). To compare metabarcoding results and seine net surveys, additional shrimp, sediment and water samples were collected from 2 sites in the Tweed and Tees estuaries in the UK, in May-June 2017 (Figure 6.1A). At these two sites, beach seine net surveys were carried out at low tide (±3 h) to assess fish catch data and for the collection of shrimp for stomach extractions. Seine net surveys were conducted by the Environmental Agency as part of a larger collaborative study, using a multi-method approach to monitor fish diversity in UK estuaries, following WFD-UKTAG (2014) and Colclough et al. (2002). Surface water samples (0-1 m depth) were collected in sterile 2L bottles provided with a 200 µm nylon mesh. All samples were packed in individual plastic bags and placed on ice for transport and stored at -20 °C. Prior to transport, sediment samples were conserved in 96 % ethanol.

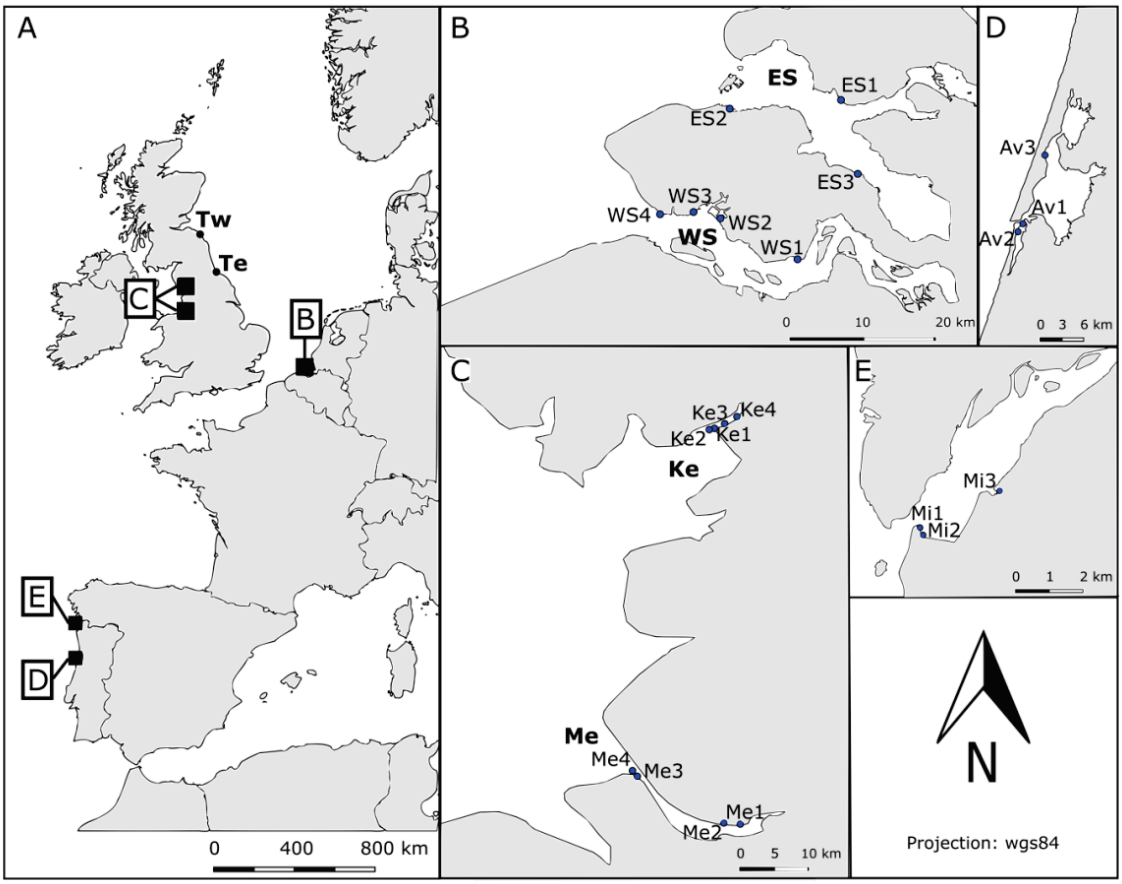

Figure 6.1. Overview of sample locations, illustrating (A) the overall western European scale, including the location of the Tweed (Tw) and Tees (Te) estuaries; (B) the Dutch estuaries, Western Scheldt (WS) and Eastern Scheldt (ES); the British estuaries (C), Mersey (Me), Kent (Ke) (with Tees and Tweed only in inset A); the Aveiro (D) and Minho (E) estuaries in Portugal. Small numbered dots within estuaries represent individual collection points for shrimp and sediment samples. Source map: OpenStreetMap.

6.3.2. DNA extraction

In total, 483 stomachs (Appendix 6.1) were dissected using flame sterilised tools from shrimp which had a visually full stomach. Up to eight full stomachs were pooled per sample, in order to reduce randomness (Deagle et al., 2005, Ray et al., 2016), prior to DNA extraction, resulting in 3 samples per site. Three sites (Av3, Me4 and Ke2) contained only two samples due to low number of shrimp caught with full stomachs at these locations (see Chapter 4). Water samples (0.9 L) were filtered using Sterivex filter units (0.22 µm pore size; Merck Millipore) upon arrival to the laboratory (within 4 hours after collection). Pooled stomach (0.25 g) and sediment (10 g) samples were homogenised and DNA extracted using the PowerSoil® DNA isolation Kit (Mo-Bio laboratories) and the PowerMax® DNA Soil Kit (Mo-Bio laboratories) respectively. For the water samples, DNA was extracted from the filters and

isolated using the DNeasy PowerWater® DNA isolation Kit (Qiagen). A Qubit fluorometer (Thermo-Fisher Scientific) was used to assess the DNA concentrations of the purified extracts. DNA extraction and pre-PCR preparations were performed in separate labs from post-PCR procedures to avoid contaminations.

6.3.3. DNA amplification and high-throughput sequencing

For the evaluation of *C. crangon* stomach contents to assess fish diversity on a European scale, stomach and sediment sample extracts from the Dutch, Portuguese and UK (except Tweed and Tees) estuaries were amplified using two primer sets: Leray-XT (COI; Wangensteen et al., 2017 in review) and MiFish (12S; Miya et al., 2015).

The Leray-XT primer set targets the 313-bp Leray fragment (Leray et al., 2013) of the mitochondrial cytochrome *c.* oxidase subunit I (COI) region and amplifies a broad range of taxa including most metazoan and other eukaryotic groups (Wangensteen et al 2017 in review). The Leray-XT primer set uses mlCOIintF-XT primer (5'-GGWACWRGWTGRACWITITAYCCYCC-3') as forward primer (Leray et al., 2013; Wangensteen et al., in review) and jgHCO2198 (5'-TAIACYTCIGGRTGICCRAARAAYCA-3'; Geller et al., 2013) as reverse primer. The PCR amplification and subsequent HT-sequencing of this amplicon was conducted as part of a larger project describing the diet of *C. crangon* in Chapter 4.

The MiFish primer set (Miya et al., 2015) has been developed to target a hypervariable region in the mitochondrial 12S rRNA gene (163–185 bp) and specifically amplifies fish and other vertebrate DNA. COI and 12S are commonly used markers for fish metabarcoding studies (Shaw et al., 2016, Deagle et al., 2014). Both primer pairs target short fragments of mitochondrial multi-copy genes, which are preferred for environmental samples due to general low quality of envDNA (Calvignac-Spencer et al., 2013a). For the specific comparison of sample type efficacy, stomach, sediment and water sample extracts from the Tweed and Tees estuaries were amplified using the MiFish primer set only.

The PCR mix recipe for the Leray-XT primer set included 10 µl AmpliTaq gold 360Master mix (Applied Biosystems), 3.2 µg Bovine Serum Albumin (Thermo Scientific), 1 µl of each of the 5 µM forward and reverse tagged-primers (including 2-4 leading Ns and 8-bp sample tags), 5.84 µl H_2O and 2 µl extracted DNA template (~ 5 ng μl^{-1}). The PCR profile included an initial

denaturing step of 95 °C for 10 min, 35 cycles of 94 °C for 1 min, 45 °C for 1 min and 72 °C for 1 min and a final extension step of 72 °C for 5 min. PCR products (including 2 negative controls) with sample tags attached were pooled into two multiplex sample pools (sediment sample pool and stomach sample pool) and purified using MinElute columns (Qiagen). Library preparation was performed using the NextFlex PCR-free library preparation kit (BIOO Scientific) and library quantification was done using the NEBNext qPCR quantification kit (New England Biolabs). Libraries were pooled (along with 0.7% PhiX v3, Illumina, serving as a positive sequencing quality control) in a 1:4 sediment:stomach molar concentration ratio (reflecting the sediment:stomach sample ratio) and sequenced (final molarity of 8 pM) on an Illumina MiSeq platform using v2 chemistry (2x250 bp paired-ends).

Prior to the 12S-MiFish PCR amplification, DNA from the three stomach extractions per site was pooled before PCR amplification, resulting in final pools of 16-24 stomachs per sample (with the exception of the Tweed and Tees samples; see appendix 6.1) and standardised to ~ 5 ng μl^{-1}. Amplification of the 12S-MiFish fragment (for the sediment, water and pooled stomach samples) was achieved using a two-step PCR protocol by first amplifying the amplicon using untagged primers and sequentially amplifying the product of the first PCR with tagged primers to attach a 7-bp index to each sample (Miya et al., 2015, Andruszkiewicz et al., 2017). Between the two PCR steps, a size selection was performed using MultiScreen® $PCR_{\mu 96}$ plates (Millipore) to remove any leftover primers. The MiFish primer pair was used for both PCR steps (forward: 5'-GTCGGTAAAACTCGTGCCAGC-3'; reverse: 5'-CATAGTGGGGTATCTAATCCCAGTTTG-3'; Miya et al., 2015) and the PCR mix recipe was the same as the one mentioned above for the COI amplification. The PCR profile (for both steps) included an initial denaturing step of 95 °C for 10 min, 40 cycles of 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 30 sec and a final extension step of 72 °C for 5 min. Two technical PCR replicates were produced per sample and two negative controls were included. PCR products were pooled into two multiplex sample pools (one pool per PCR replicate) and the pools were sequenced in equimolar concentrations (final molarity of 9 pM) along with 0.8% PhiX on Illumina MiSeq platform using v2 chemistry (2x150 bp paired-ends) in accordance to the protocol described above for the COI fragment.

6.3.4. Bioinformatic and data analyses

Bioinformatic analyses were performed using the OBITools metabarcoding software suite (Boyer et al., 2016). Assessment of read quality was done with FastQC, paired-end read alignment with illuminapairedend, and reads with alignment quality score > 40 were retained. Demultiplexing and primer removal was achieved using ngsfilter. Aligned reads with a length of 303-323 bp (for COI) or 140-190 (for 12S) and free of ambiguous bases, were selected using obigrep and dereplicated with obiuniq. Chimeras were removed using the uchime-denovo algorithm (implemented in VSEARCH; Edgar et al., 2011, Rognes et al., 2016). Amplicon clustering was performed using the SWARM 2.0 algorithm (Mahé et al., 2015, Mahé et al., 2014) with a d value of 13 for the COI pipeline (equivalent to 95% sequence identity for the Leray fragment), and with a d value of 3 for the 12S pipeline (equivalent to 98% sequence identity for the Miya fragment). Taxonomic assignment was achieved using the ecotag algorithm (Boyer et al., 2016) on representative sequences for each MOTU to taxa in relation to local reference databases (Wangensteen et al., in review). The COI database (db COI Sep2017) contained 191,295 filtered COI sequences of eukaryota retrieved from the BOLD database (Ratnasingham and Hebert, 2007) and the EMBL repository (Kulikova et al., 2004). The 12S database (db Miya Sep2017) contained 6,868 sequences from vertebrates retrieved from Genbank. Further refinement of the data was achieved by clustering MOTUs assigned to the same species, the application of abundance renormalization to remove false positives arising from tag switching (Wangensteen and Turon, 2016), and the removal of singletons. Reads not belonging to fish taxa (Actinopterygii and Agnata) were removed. To avoid false positives and remove low frequency noise, a minimum copy threshold of 5 reads per sample was applied to the COI dataset (Alberdi et al., 2018; Chapter 4). False positives were removed from the 12S dataset by means of a restrictive approach in which only MOTUs that occurred in both PCR replicates were considered (Alberdi et al., 2018). Remaining reads of the two PCR-replicates were merged per sample after filtering. Both the minimum copy threshold of 5 copies and the restrictive PCR-replicate approach can be considered conservative and may have had a negative effect on the detected diversity (Alberdi et al., 2018). Stomach reads were merged per site for the COI dataset to obtain comparable datasets between the two markers, based on the same stomachs pools. An overview of the pipelines is reported in appendix 6.2

Statistical analyses were performed in R v3.1.3 (https://www.R-project.org/) with the packages vegan (v2.3-5; Oksanen et al., 2016) and BiodiversityR (v2.5-3; Kindt and Coe, 2005). Differences in the mean number of bony fish MOTUs per sample between markers and sample media were analysed using Wilcoxon signed rank tests. Non-metric multidimensional scaling (nMDS) was used to visualise differences between estuaries and sites based on Jaccard dissimilarities. Multivariate analyses were conducted based on presence-absence data (acquired with the MiFish primers) using the PERMANOVA functions adonis and nested.npmanova (Jaccard dissimilarities and 1000 permutations) to test for differences in community structure between sample types, countries and estuaries (nested within countries). MOTU richness was represented as MOTU/species accumulation curves to illustrate differences between markers, sample substrates and countries. Generalised linear models (with Poisson-distribution modelling of residuals) were constructed to test the effects of country, estuary (nested within countries), and the number of pooled stomachs on the MOTU richness per sample.

6.4. Results

6.4.1. Molecular biodiversity assessment

Total numbers of 2,060,514 and 4,997,391 reads were obtained from 12S-MiFish and COI-Leray-XT amplifications respectively after demultiplexing, quality and sequence-length filtering. The MiFish primers only amplified chordates (Actinopterygii, Agnatha, Aves, Mammalia) while the Leray-XT primer pair amplified 40 phyla (Chapter 4). Percentage of fish (Actinopterygii) reads was high for the MiFish primers (75-89%) and low for the Leray-XT primers (sediment: <0.01%, stomach: 7%; table 6.1). Taxonomic assignment resulted in a total of 219 Actinopterygii MOTUs identified using the MiFish marker, of which 62 were identified to the species or genus level. Of the 27 Actinopterygii MOTUs detected in the samples amplified with the Leray-XT marker, 25 were assigned to the species or genus level. Only 1 fish MOTU (*Dicentrarchus labrax*; 15 reads) was amplified with the Leray-XT primers from sediment samples. This MOTU was, furthermore, only detected at one site (Minho 1) and was not considered for further analyses. Figure 6.2 shows a heat map of all MOTUs identified to the species or genus level with the different markers, sample locations and sample media. Large variation is visible in the species detected across samples. Notable observations are: the detection of the river lamprey *Lampetra fluviatilis* in the Eastern Scheldt; the wide presence of *D. labrax*, independent of marker and sample media; the wide occurrence and relatively high abundances of *Pleuronectes* sp., *Salmo trutta*, *Scomber scombrus* and *Trachurus trachurus* in samples amplified with MiFish primers but not with Leray-XT; and the complementary power of both markers to identify some taxa to the species level (e.g. *Atherina* sp. (Mifish) is most likely *A. presbyter* (Leray-XT) in Aveiro 1).

Table 6.1. Total number of bony fish (class Actinopterygii), lamprey (class Petromyzonti) and non-fish reads detected in samples sequenced using Leray-XT (COI) and MiFish (12S) primer pairs. Fish reads are given after quality filtering and removal of false positives.

Reads	**Leray-XT**		**MiFish**		**Tees & Tweed (MiFish)**		
	Sediment	Stomach	Sediment	Stomach	Sediment	Stomach	Water
Bony fish	15	306,997	407,377	799,272	199,254	205,886	119,652
Lamprey	0	0	0	63	28	0	0
Non-fish	620,310	4,070,069	107,059	95,720	60,574	26,797	38,923
Total	620,325	4,377,066	514,436	895,055	259828	232683	158575
Fish reads (%)	0.002%	7.01%	79.19%	89.31%	76.69%	88.44%	75.46%

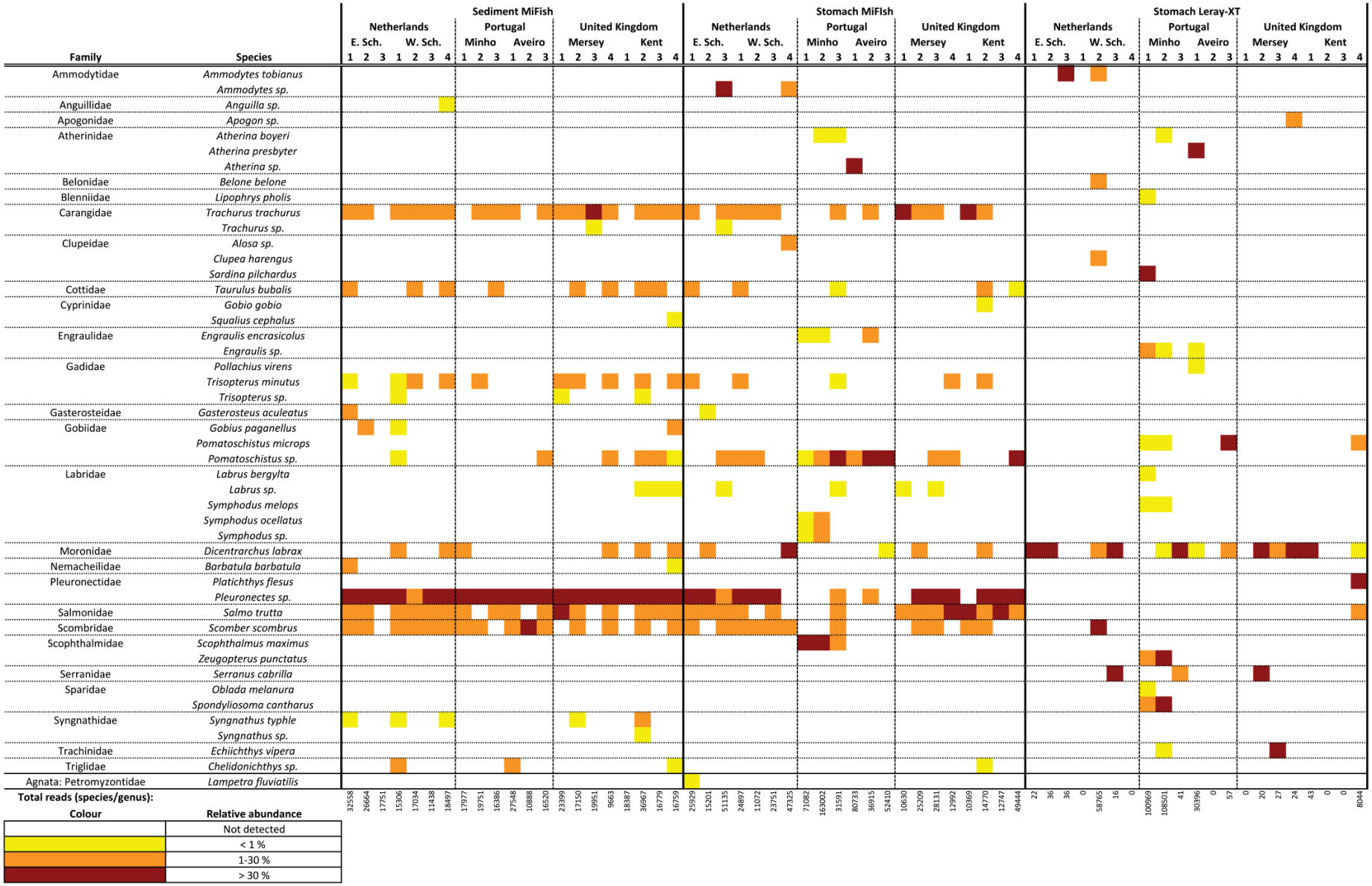
Sediment MiFish
Stomach MiFIsh
Stomach Leray-XT
Netherlands
Portugal
United Kingdom
E. Sch.
W. Sch.
Minho
Aveiro
Mersey
Kent
Family
Species
Ammodytidae
Ammodytes tobianus
Ammodytes sp.
Anguillidae
Anguilla sp.
Apogonidae
Apogon sp.
Atherinidae
Atherina boyeri
Atherina presbyter
Atherina sp.
Belonidae
Belone belone
Blenniidae
Lipophrys pholis
Carangidae
Trachurus trachurus
Trachurus sp.
Clupeidae
Alosa sp.
Clupea harengus
Sardina pilchardus
Cottidae
Taurulus bubalis
Cyprinidae
Gobio gobio
Squalius cephalus
Engraulidae
Engraulis encrasicolus
Engraulis sp.
Gadidae
Pollachius virens
Trisopterus minutus
Trisopterus sp.
Gasterosteidae
Gasterosteus aculeatus
Gobiidae
Gobius paganellus
Pomatoschistus microps
Pomatoschistus sp.
Labridae
Labrus bergylta
Labrus sp.
Symphodus melops
Symphodus ocellatus
Symphodus sp.
Moronidae
Dicentrarchus labrax
Nemacheilidae
Barbatula barbatula
Pleuronectidae
Platichthys flesus
Pleuronectes sp.
Salmonidae
Salmo trutta
Scombridae
Scomber scombrus
Scophthalmidae
Scophthalmus maximus
Zeugopterus punctatus
Serranidae
Serranus cabrilla
Sparidae
Oblada melanura
Spondyliosoma cantharus
Syngnathidae
Syngnathus typhle
Syngnathus sp.
Trachinidae
Echiichthys vipera
Triglidae
Chelidonichthys sp.
Agnata: Petromyzontidae
Lampetra fluviatilis
Total reads (species/genus):
32558 26664 17751 15306 17034 11438 18497
17977 19751 16386 27548 10888 16520
23399 17150 19951 9663 18387 36967 16779 16759
25929 15201 51135 24897 11072 23751 47325
71082 163002 31591 80733 36915 52410
10630 25209 28131 12992 10369 14770 12747 49444
22 36 36 0 58765 16 0
100969 108501 41 30396 0 57
0 20 27 24 43 0 0 8044
Colour
Relative abundance
Not detected
< 1 %
1-30 %
> 30 %

Figure 6.2 (*previous page*). Heatmap fish species and genera detected in samples taken from Dutch (E.Sch: Eastern Scheldt & W.Sch: Western Scheldt), UK (Mersey & Kent) and Portuguese (Minho & Aveiro) estuaries. Fish taxa detected in Crangon crangon pooled stomach samples is shown after DNA amplification with two markers: MiFish (12S) and Leray-XT (COI). Sediment samples are only shown after DNA amplification with the MiFish primer pair since Leray-XT amplification resulted in the detection of only 1 MOTU (D. labrax) in 1 sample (Minho 1). Colours represent differences in relative read abundances and the numbers below the columns shows the total number of fish reads per sample that could be assigned to the species or genus level.

6.4.2. Comparison of 12S and COI molecular markers and sample types

MOTU accumulation curves showed no differences in the total number of MOTUs identified to the species or genus levels between markers (MiFish/Leray-XT) and sample types (sediment/stomach) (Figure 6.3), except for the Leray-XT sediment samples (only 1 MOTU detected). Venn diagrams showed large overlap between markers in the fish families detected, but differences were noted in the species identified (Figure 6.4A). The total number of fish MOTUs per stomach sample was significantly higher in samples amplified with MiFish than Leray-XT when identified to the family or genus level, but did not differ significantly when only MOTUs identified to the species level were considered (Table 6.2).

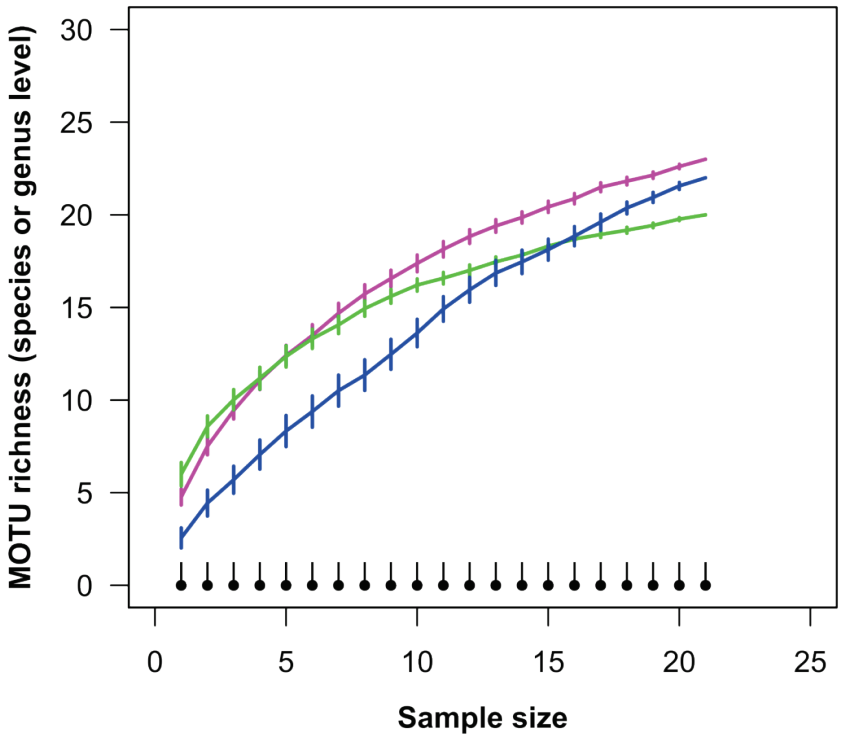

Figure 6.3. MOTU accumulation curves (±SE; 100 permutations) representing the number of bony fish MOTUs identified to the species or genus level detected in sediment and *Crangon crangon* pooled stomach samples analysed with two different primer pairs. Green: Sediment - MiFish (12S); magenta: Stomach - MiFish; black: Sediment - Leray-XT (COI); blue: Stomach - Leray-XT. Sediment - Leray-XT values are estimated since only 1 MOTU was detected.

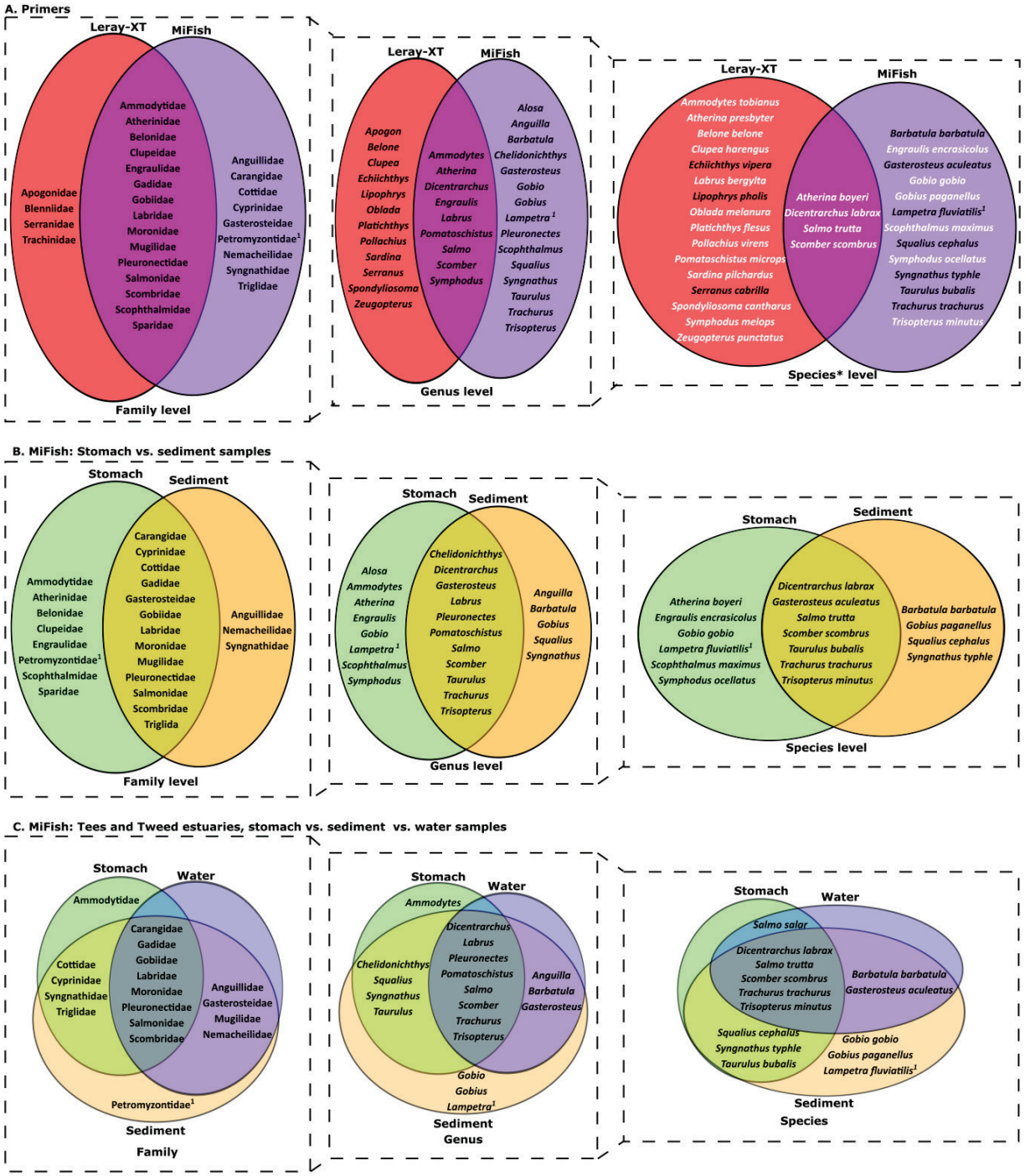

Figure 6.4. Venn diagrams of fish families, genera and species detected in the DNA of (A) *Crangon crangon* stomach pooled samples amplified with two different primer pairs: MiFish (12S) and Leray-XT (COI); (B) Crangon crangon pooled stomach and sediment samples amplified with MiFish (12S) primers;(C) DNA amplified with MiFish (12S) primers of *Crangon crangon* pooled stomach, sediment and water samples collected in the Tees and Tweed estuaries in the UK. * Species for which the family was detected with both markers are indicated in white**;** 1: Class Petromyzonti

Almost half of the fish taxa identified with the MiFish marker were observed in both sample types and this proportion of taxa remained fairly constant among the different taxonomic levels considered (Figure 6.4B). The total number of taxa identified per MiFish-amplified sample did not differ significantly between sediment and stomach samples, independently of the taxonomic level of interest (Table 6.3).

Table 6.2. Differences in mean number of bony fish MOTUs per sample identified to different taxonomic levels in *Crangon crangon* pooled stomach samples amplified with MiFish (12S) and Leray-XT (COI) primer pairs. Higher taxonomic ranks include MOTUs identified to the lower levels.

	Mean ± SE number of MOTU per sample		**Wilcoxon signed rank test**		
	Leray-XT stomach	**MiFish stomach**	**N**	**V**	**P**
All MOTUs	2.2 ± 0.6	6.6 ± 0.6	21	8.0	<0.001*
Family level	2.2 ± 0.6	5.8 ± 0.6	21	13.5	0.001*
Genus level	2.2 ± 0.6	4.9 ± 0.5	21	22.5	0.004*
Species level	2.0 ± 0.5	3.0 ± 0.4	21	76.5	0.177

Table 6.3. Differences in mean number of bony fish MOTUs per sample, amplified with 12S-MiFish primers, and identified to different taxonomic levels between *Crangon crangon* pooled stomach and sediment samples. Higher taxonomic ranks include MOTUs identified to the lower levels.

	Mean ± SE number of MOTU per sample		**Wilcoxon signed rank test**		
	MiFish stomach	**MiFish sediment**	**N**	**V**	**P**
All MOTUs	6.6 ± 0.6	7.0 ± 0.7	21	82.0	0.61
Family level	5.8 ± 0.6	6.9 ± 0.8	21	68.0	0.17
Genus level	4.9 ± 0.5	5.9 ± 0.7	21	66.0	0.15
Species level	3.0 ± 0.4	4.0 ± 0.5	21	51.5	0.14

More fish species were detected with metabarcoding using the MiFish primer pair than by traditional seine net surveys in the Tees and Tweed estuaries (Table 6.4). In the Tees estuary, 18 fish taxa (nine of which assigned to the species level) were detected using molecular methods while only five taxa (all identified to the species level) during the seine net survey. Although no MOTUs were detected by metabarcoding that could be assigned to these five species, taxa were assigned to the same family or genus level as four of these species (with the exception of *Ammodytes tobianus*). Nine species and four higher taxa (family or genus) were exclusively detected by metabarcoding. In the Tweed estuary, 23 fish taxa (of which 14 assigned to the species level) were detected using molecular methods and only six taxa (all identified to the species level) during the seine net survey. Three species were detected by both methods. Another three species were detected exclusively by netting, but these matched taxa that were assigned to the same family or genus by metabarcoding. Eleven

species and six higher taxa (family or genus) were exclusively detected by metabarcoding, including the lamprey *Lampetra fluviatilis,* in the tweed.

Water, sediment and *C. crangon* stomach samples collected from the Tees and Tweed estuaries showed extensive overlap in the species, genera and families detected (Figure 6.4C). Although some genera were exclusively detected in either sediment (*Gobio*, *Gobius* and *Lampetra*) or stomach (*Ammodytes*) samples, no species, genera or families were solely detected in water samples.

Table 6.4. Fish species detected by 12S metabarcoding and concurrent seine net surveys. Metabarcoding results are based on combined data from sediment, water and *C. crangon* stomach samples amplified with the MiFish primer pair (see Figure 6.4C). Percentage detected shows the percentage of species identified per estuary and, in brackets, the inferred percentage of species detected if MOTUs identified to the family or genus level are included.

		Tees estuary		Tweed estuary	
Family	**Species**	**Metabarcoding**	**Netting**	**Metabarcoding**	**Netting**
Ammodytidae	*Ammodytes tobianus*		√	[1]	
Anguillidae	*Anguilla* sp.	[1]		[1]	
Carangidae	*Trachurus trachurus*	√		√	
Clupeidae	*Clupea harengus*	[2]	√	[2]	√
	Sprattus sprattus	[2]	√		
Cottidae	*Taurulus bubalis*	√		√	
Cyprinidae	*Gobio gobio*			√	
	Squalius cephalus			√	
Gadidae	Unassigned			[2]	
	Trisopterus minutus	√		√	
Gasterosteidae	*Gasterosteus aculeatus*	√		√	√
Gobiidae	*Gobius paganellus*			√	
	Pomatoschistus minutus	[1]		[1]	√
Labridae	*Labrus* sp.			[1]	
Moronidae	*Dicentrarchus labrax*	√		√	
Mugilidae	Unassigned	[2]		[2]	
Nemacheilidae	*Barbatula barbatula*	√		√	
Petromyzontidae*	*Lampetra fluviatilis**			√	
Pleuronectidae	*Platichthys flesus*	[2]	√	[2]	√
	Pleuronectes platessa	[1]	√		
Salmonidae	*Salmo salar*			√	√
	Salmo trutta	√		√	√
Scombridae	*Scomber scombrus*	√		√	
Syngnathidae	*Syngnathus typhle*	√		√	
Triglidae	*Chelidonichthys* sp.	[1]		[1]	
% Detected		**50 (94)**	**28**	**61 (100)**	**26**

[1] Assigned to genus level; [2] Assigned to family level; * Class Petromyzonti.

6.4.3. Geographical differences

MOTU accumulation curves per country showed relatively high MOTU richness in stomach samples from Portugal and the Netherlands (Figure 6.5A), when amplified with the MiFish primer set. These differences were, however, not apparent when only MOTUs identified to the species or genus level were considered (Figure 6.5B). Stomach samples from Portugal showed a higher MOTU and species/genus richness than samples from the Netherlands and the UK (Figure 6.5C, D) when amplified with the Leray-XT primer set.

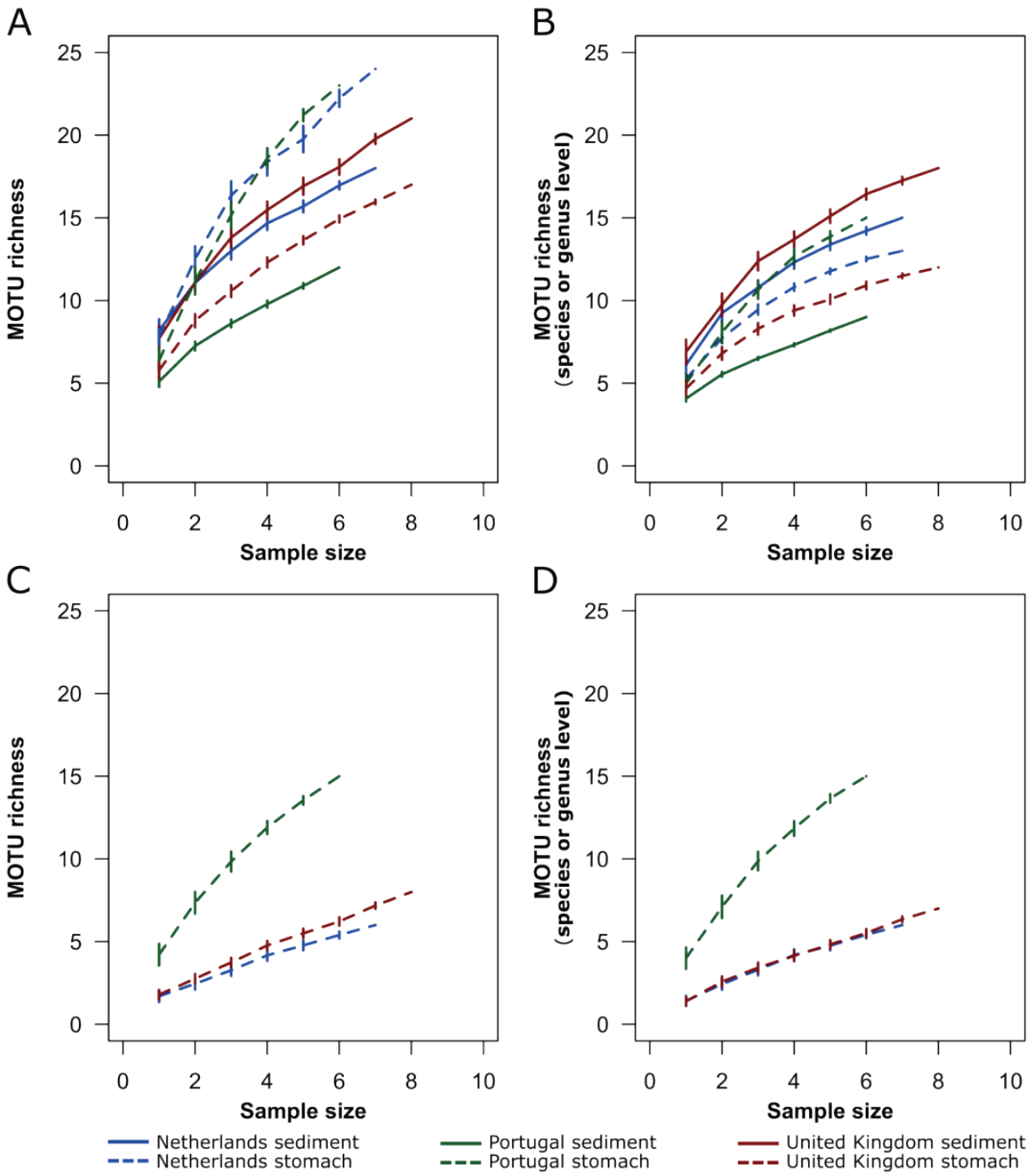

Figure 6.5. MOTU accumulation curves (±SE; 100 permutations) for the number of bony fish MOTUs (A-C) and taxa identified to the species or genus level (B-D) detected in DNA extracted from sediment and *Crangon crangon* pooled stomach samples collected in different countries. Samples were analysed with two different primer pairs: 12S MiFish (A,B); COI Leray-XT (C,D).

Median MOTU richness per estuary is shown in Figure 6.6. The number of MOTU detected with the MiFish marker in the stomach samples was not significantly influenced by the number of pooled stomachs (coefficient ± SE = 0.02 ± 0.04; Z = 0.57, P = 0.567) and did not differ between countries (Appendix 6.3). At the estuary (within country) level (Figure 6.6A), only the Minho estuary showed a significant positive effect on the model outcome (coefficient ± SE = 1.01 ± 0.39; Z = 2.56, P <0.05; Appendix 6.3). There was no geographical variation detected in MOTU richness from the sediment samples amplified with the Miya primer set either at the country or at the estuary level (Figure 6.6B; Appendix 6.4). Also, the MOTU richness of the stomach samples amplified with the Leray-XT marker did not show any geographical variation or was influenced by the number of stomachs per pool (Figure 6.6C; Appendix 6.5). Independently of the marker or sample type, MOTU richness estimations showed large distribution of data at several estuaries (Figure 6.6). This indicates large variation between sites within estuaries which could mask overall geographical differences.

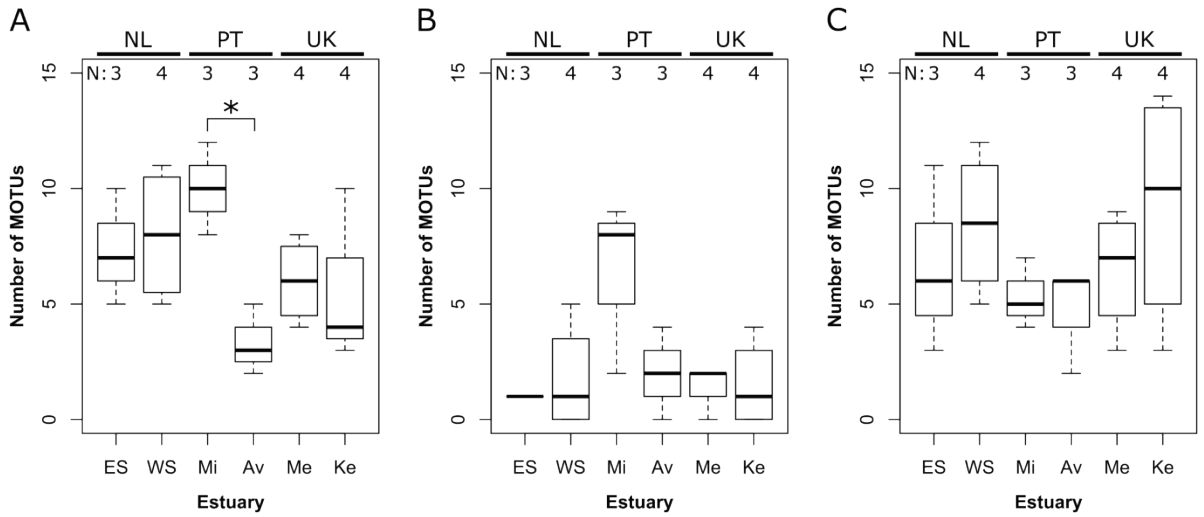

Figure 6.6. Boxplots showing differences in bony fish MOTU richness per estuary for (A) *Crangon* crangon stomach samples and (B) sediment samples amplified with 12S MiFish primer pair, and (C) *C. crangon* stomach samples amplified with COI Leray-XT primer pair. * P < 0.05. Estuaries: Eastern Scheldt (ES); Western Scheldt (WS); Minho (Mi); Aveiro (Av); Mersey (Me); Kent (Ke).

Multivariate analysis of the fish community detected with the MiFish primers showed extensive overlap between samples collected in different estuaries, as well as similarities between stomach and sediment samples (Figure 6.7A). PERMANOVA analysis shows significant differences in community structure between sample types (pseudo-F = 2.14, P < 0.05) but no significant differences between estuaries (pseudo-F = 1.10, P = 0.26). Separate analyses per sample type showed that the community structure did not differ significantly between countries and estuaries (nested within countries) for either sediment (country: pseudo-F = 1.11, P = 0.39; estuary in country: pseudo-F = 0.99, P = 0.85) or stomach samples (country: pseudo-F = 2.27, P = 0.09; estuary in country: pseudo-F = 2.50, P = 0.54).

Multivariate analysis of the fish community detected with the Leray-XT primers in *C. crangon* stomach samples also showed overlap between samples collected in different estuaries (Figure 6.7B). PERMANOVA analysis did not show significant differences in community structure between countries (pseudo-F = 1.35, P = 0.22) and estuaries nested within countries (pseudo-F = 1.62, P = 0.63). Six sites were removed from the Leray-XT analysis because they did not yield any fish reads (see Figure 6.2). Though no significant differences were detected, visual inspection of the nMDS diagrams (Figure 6.7) showed that the Portuguese estuaries had a slightly greater spatial heterogeneity compared to the other estuaries.

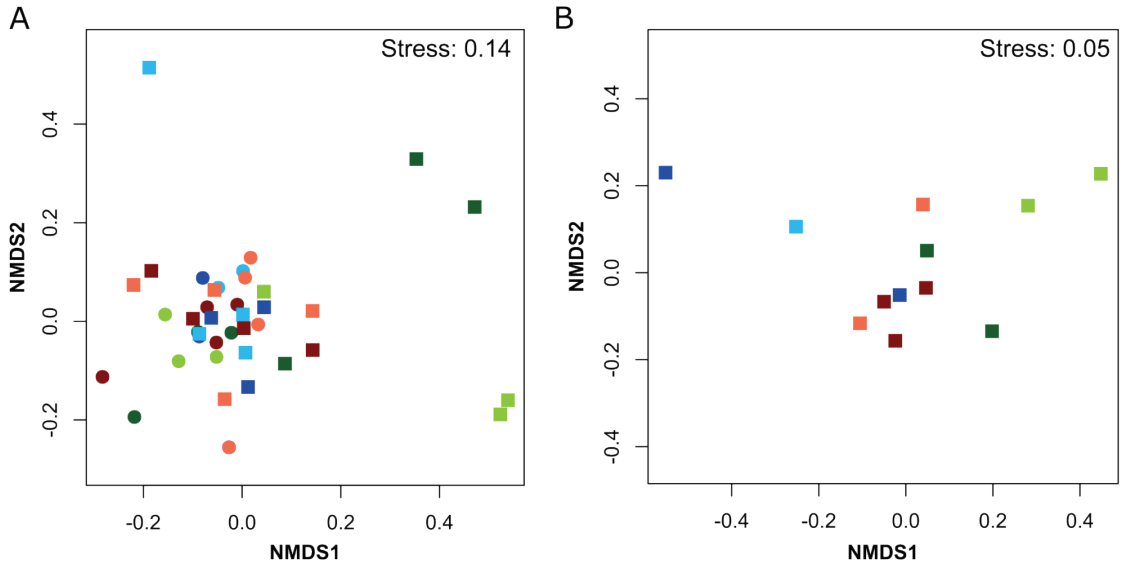

Figure 6.7. Multidimensional scaling analysis (based on Jaccard dissimilarities) of bony fish MOTUs detected in sediment (dots) and *Crangon crangon* pooled stomach samples (squares), after DNA amplification with (A) MiFish (12S) and (B) Leray-XT (COI) primer pairs. Estuaries are identified by colour. Netherlands: Eastern Scheldt (dark blue), Western Scheldt (light blue); Portugal: Aveiro (dark green), Minho (light green); UK: Kent (light red), Mersey (dark red).

6.5. Discussion

Estuaries are under substantial anthropogenic pressures, including fisheries, pollution, shipping and the spread of invasive species, thus the monitoring of their ecological status and variation is essential to safeguard ecosystem functioning and the services provided (Sheaves et al., 2015, Martínez et al., 2007). To improve fish diversity assessment, recent studies have employed molecular tools such as envDNA metabarcoding (Evans and Lamberti, 2017, Thomsen et al., 2012). The present study introduces a novel approach, which benefits from the 'natural sampling' properties of a generalist predator/scavenger and the power and speed afforded by metabarcoding.

Results show that metabarcoding of environmental and trophic samples was much more effective in determining the local fish community structure than traditional seine net surveys, in line with a growing body of work on the use of envDNA in fish surveys (e.g. Valentini et al., 2016, Thomsen et al., 2012). Twice the number of species and more than three times the number of taxa (assigned to the genus of family level) were detected using 12S-metabarcoding of a combination of sediment, shrimp stomach and water samples compared to concordant seine net surveys. Furthermore, fish taxa detected by metabarcoding, but not by traditional netting, included some important taxa for conservation such as *Anguilla* and *Lampetra*. Although it was not possible to identify all species caught during the seine net surveys at the species level using molecular assignment only, the family/genus-level identification indicates that the DNA of these species was indeed amplified, hence allowing in most cases indirect inference on species presence. Improvements of the reference database or marker's taxonomic resolution will be required to attain unambiguous, direct molecular identification of these taxa at the species level (Shaw et al., 2016, Alberdi et al., 2018).

The fish diversity detected during this study, by using a combination of sediment and *C. crangon* stomach samples, reflects a typical European estuarine community, including estuarine residents (e.g. *A. tobianus* and *Pomatoschistus microps*) and species that use estuaries as a nurseries and/or feeding grounds (e.g. *D. labrax* and *Pleuronectes platessa*), migrate through them (e.g. *Anguilla* sp. and *Salmo trutta*) or behave as marine or freshwater stragglers (e.g. *Scomber scombrus*, *Trachurus trachurus* and *Gobio gobio*) (Elliott et al., 2007, Elliott and Dewailly, 1995, Maes et al., 2005). Several species detected, such as eel (*Anguilla* sp.), European plaice (*P. platessa*), sea bass (*D. labrax*) and Atlantic mackerel (*S. scombrus*)

are important commercial targets. Considering the small size of *C. crangon* caught (20-50 mm TL; Chapter 4), it is surprising to see that such a small shrimp feed on a large range of fish species including several known and potential own predators (e.g. *P. microps and D. labrax*; (Cattrijsse et al., 1997). Consumption of fish tissue is, therefore, likely a combination of scavenging on adults and direct predation on juveniles/larvae (van der Veer and Bergman, 1987, Ansell et al., 1999; Chapter 4). Although soft bottom habitats were sampled, several hard-bottom associated species were detected (e.g. *Lipophrys pholis* and *Labrus bergylta*) which could have been occasional visitors from nearby rocky shores (e.g. rocky outcrops located near the mouth of the Minho estuary) or were captured/scavenged by shrimp migrating in and out the estuaries (Al-Adhub and Naylor, 1975). Besides DNA originated from the biota present in the estuaries, DNA detected in the sediment and water samples might also have been transported *post mortem* from other adjacent areas by river runoff or during tidal movements (Barnes and Turner, 2016).

6.5.1. Geographical variation

No clear geographical patterns were detected in fish community structure or MOTU richness between the countries and estuaries studied. Although the Minho estuary showed a higher fish richness than the other estuaries, this could be an artefact caused by the higher small-scale habitat heterogeneity of the locations sampled in this estuary. The Minho estuary has been reported to hold low epibenthic diversity due to a high fresh water discharge and salinity variations (Costa-Dias et al., 2010). Two out of three locations sampled were close to the estuary mouth and showed a high number of marine straggles, possibly inflating the observed diversity compared to the other estuaries in which the sample locations were more equally distributed. European estuaries show high similarities in fish assemblages (especially for very common/abundant species; Elliott and Dewailly, 1995), with patchy, small-scale heterogeneity, and seasonal changes often greater than geographic variation (Jovanovic et al., 2007). Furthermore, several studies show that detectability of envDNA depends on a range of factors, which complicate the acquisition of a comprehensive overview of the diversity of a system. These factors include DNA dispersion and dilution (Barnes and Turner, 2016), the number of biological replicates and the amount of substrate sampled (Andruszkiewicz et al., 2017, Shaw et al., 2016, Taberlet et al., 2012b). Samples taken within the same estuary showed large variation in fish communities which might have masked

overall geographical differences. Consequently, the limited number, and temporal range of the samples used during this study might not have been sufficient to show geographical patterns on a European scale. Further studies with expanded geographical range and seasonal samplings are likely to yield the full breadth of European estuarine ichthyofaunal complexity.

6.5.2. Marker and DNA medium choice

The results of this study add to the growing body of evidence underpinning the efficacy of molecular tools to effectively detect biodiversity (Evans and Lamberti, 2017, Andruszkiewicz et al., 2017, Taberlet et al., 2012a), but also show that a combination of multiple markers and different sample types are required to gain a comprehensive understanding of the study system (Deagle et al., 2014, Shaw et al., 2016, Alberdi et al., 2018). Environmental DNA is generally only present in trace amounts in the environment and the concentration of DNA can differ between media, influencing the detectability of taxa (Shaw et al., 2016, Turner et al., 2015, Taberlet et al., 2012b). No major differences were detected in the number of fish species identified with the MiFish primer pair between the sediment samples and *C. crangon* stomach contents. In the samples from the Tees and Tweed estuaries, more fish species were detected from the envDNA extracted from the sediment than from the water and no species, genera or families were exclusively detected in the water samples. Differences in fish detectability between these sample types could be due to the generally higher concentration and temporal persistence of DNA in sediment samples compared to the water column (Turner et al., 2015) or caused by differences in the volumes of substrates used (Shaw et al., 2016).

The differences in species detection between the 12S and COI markers is likely due to a combination of primer bias, differences in reference database completeness, and the taxonomic resolution of the markers (Alberdi et al., 2018, Taberlet et al., 2012a). Additionally, small variation in processing of samples amplified with different markers (pooling, bioinformatics pipelines) could have influenced the results (Alberdi et al., 2018), but these effects are assumed to be negligible compared to the factors mentioned above. Of the markers used during this study, COI was more accurate for species-level detection than 12S, due to its better taxonomic resolution, which ensures high discrimination power at the

species level, and the availability of an exhaustive and well- curated reference database (BOLD; Ratnasingham and Hebert, 2007). Its use was, however, severely limited in samples where the relative amount of fish DNA was low (e.g. sediment samples) due to the nearly universal taxonomic breadth of the COI primers used (Wangensteen et al., in review). Interestingly, more teleost DNA was amplified with Leray-XT from the stomachs samples than from the sediment samples, even though no blocking primers were used to block *C. crangon* DNA (Ray et al., 2016). The primer-binding sites of COI-based markers are, usually, less conserved than those of other markers such as 12S and 16S, resulting in a higher proportion of taxa potentially remaining undetected (Deagle et al., 2014). Nevertheless, the high degeneration rates of the Leray-XT primer set contributed to solve this problem (Wangensteen et al., in review). The main limitations of 12S-based markers are that 12S rDNA teleost coverage is relatively poor in the NCBI nucleotide database (Andruszkiewicz et al., 2017) and the fact that 12S-based markers have lower taxonomic resolution for fish species than other markers (Pesole et al., 1999, Shaw et al., 2016). An appropriate choice of marker and sample medium are, therefore, intertwined and depend on the research question (e.g. rarity of the taxa of interest), the taxonomic resolution required, and the availability of resources to improve reference databases.

6.5.3. Applications in fisheries and environmental sciences

Fisheries science requires tools that provide reproducible data on species diversity, stock size and demographic information of the area under study, preferably for minimal cost and labour. Traditional methods are not always able to provide this, as results vary highly with the sampling technique used, including type of gear and depth of fishing. They are, furthermore, often expensive and labour intensive (Lapointe et al., 2011, Cotter et al., 2004, Thomsen et al., 2012, Courrat et al., 2009). Presently, environmental DNA metabarcoding techniques also show limitations for several of these requirements since they are not able to assess population structure and fish condition, nor they provide real-time and fine-scale information (Evans and Lamberti, 2017, Shaw et al., 2016). On the positive side, envDNA samples are easier to collect, require lower sampling effort and are less labour intensive than traditional fishing methods (Evans et al., 2017, Smart et al., 2016, Boyer et al., 2015). In addition, molecular monitoring of fish populations (either from environmental or stomach samples) does not require taxonomic expertise, is more objective than traditional methods

and, in general, results in more species detected than conventional methods (Thomsen et al., 2012, Valentini et al., 2016; this study). Besides species distribution assessments, the use of envDNA is especially useful for the detection and monitoring of rare (e.g. *Anguilla* and *Lampetra*) and invasive species (Evans and Lamberti, 2017), as required for compliance with environmental policy, such as the EU Habitat Directive. Since the costs of molecular consumables continues to decline and the speed of sequencing analyses and bioinformatic pipelines increases, molecular techniques (either based on environmental or stomach samples) have the potential to become a valuable complement to traditional sampling methods (Evans et al., 2017, Smart et al., 2016).

One of the appealing aspects of using a variety of media for environmental and community DNA collection is that, while these can easily be collected simultaneously, they can reveal different levels of information about the community under investigation. Environmental DNA extracted from water samples usually integrates information over large spatial scales but has a low temporal resolution due to the high dispersion and low persistence of DNA in sea water (Barnes and Turner, 2016, Thomsen et al., 2012). Sediment samples, on the other hand, can store and conserve DNA for months to years and their high spatial heterogeneity, as often detected between samples, might provide information on small spatial scales (Turner et al., 2015, Taberlet et al., 2012b). Nevertheless, estuaries act as river catchment areas and sediments store organic matter from upstream fresh-water habitats. Resuspension and horizontal transport of envDNA could thus influence interferences made from both sediment and aqueous envDNA (Turner et al., 2015, Barnes and Turner, 2016). This study is the first to bring into the scene another promising medium: the gut contents of generalist predators or scavengers. In the case of *C. crangon,* DNA extracted from its stomach contents will likely provide recent information since shrimp have a relatively fast gut passage time (4-20h; Feller, 2006, Pihl and Rosenberg, 1984, van der Veer and Bergman, 1987) and digested DNA degrades rapidly (Moran et al., 2016, Deagle et al., 2006). The area "sampled" by a pool of *C. crangon* will likely provide information on a larger spatial scale than acquired by a sediment sample since the shrimp actively moves around during night-time and shows tidal and seasonal migrations (Al-Adhub and Naylor, 1975, Henderson and Holmes, 1987, Donk and Wilde, 1981). Furthermore, the effect of resuspension and horizontal transport of upstream envDNA could be considered less influential than in other DNA media since, as a scavenger, *C. crangon* mainly consumes solid tissues which should

show a lower dispersion than extracellular envDNA. The more shrimp pooled per sample, the larger the area theoretically sampled. One of the main advantages of this method, compared to DNA extracted from other environmental sources, is that the community DNA extracted from guts will mainly represent the live community present in the system (or recently deceased in the case of scavenging) instead of the mix of cellular and extracellular DNA from different origins, which generally constitute envDNA (Barnes and Turner, 2016).

The application of gut metabarcoding has the potential of becoming a powerful tool in biodiversity assessment applications, such as in the case of bioindicators and commercially important taxa (e.g. teleosts). Although the initial results of this and other studies, using molecular or traditional techniques (Schnell et al., 2012, Lasley-Rasher et al., 2015, Boucek and Rehage, 2014), are very promising several open questions still remain only vaguely addressed. Typical envDNA aspects regarding primer bias, false positives and negatives, PCR sequencing errors, etc. (Shokralla et al., 2012, Taberlet et al., 2012a) will require further evaluation. Furthermore, no predator is ever completely opportunistic, so these studies should also include more trophic ecological issues such as predator-prey dynamics, secondary predation, and the ecology and physiology of both the predator and prey species to assess predator/prey related biases (Schnell et al., 2015b; Chapter 4, Calvignac-Spencer et al., 2013a). Nevertheless, it is clear that envDNA can be used as a bio-assessment tool for fisheries sciences to complement traditional sampling schemes, to improve species distribution assessment, and to monitor invasive and rare species, at competitive costs. The implementation of an approach that interweaves high through-put metabarcoding with the 'natural sampling capacitiy' derived from feeding activities of opportunistic/scavenging species may in the near future offer the right blend of power, speed and cost-effectiveness for large scale, routine applications.

Chapter 7.

General discussion

7.1. Main findings

Coastal habitats are some of the most productive and biodiverse ecosystems in the world and monitoring therein can provide essential information on important ecological aspects such as environmental change, animal distribution patterns, ecological networks and animal migration patterns (Martínez et al., 2007, Hyndes et al., 2014, Beck et al., 2001, Navia et al., 2016). Since these habitats are interconnected with neighbouring systems, assessing ecological patterns in these habitats is also essential for understanding environmental changes on large geographical scales (Sheaves et al., 2015, Norcross and Shaw, 1984). Predator-prey relationships play an important structuring role in coastal and estuarine communities (Van Tomme et al., 2014). The study of animal crypsis and prey selection (factors that play an essential role in predator-prey relationships), therefore, provides an in-depth understanding of the animal interactions within these systems. Furthermore, the assessment of these behaviours provides fundamental and applied knowledge about a wide range of broader subjects, including animal camouflage, speciation, environmental adaptation, visual perception, ecological networks, anthropogenic impacts and environmental policy (Chariton et al., 2015).

This thesis provides an in-depth insight on the factors playing a role in the success of *Crangon crangon* in European estuarine waters. The results show that the brown shrimp is capable of repeated fast colour adaptations to variation in background colouration, being influenced mainly by presence/absence of light and sediment colour (**chapters 2 & 3**). The occurrence of non-adaptive responses to unfavourable conditions, such as long-time exposure to a constant background colour or when prevented from burying, revealed a complex balance between behavioural-plasticity and environmental adaptation (**chapter 3**). Results also showed spatial variation in the brown shrimp's diet composition (**chapter 4**), revealing the highly flexible nature of this trophic opportunist. Its diet consisted of a wide

variety of species belonging to 35 phyla, but with a high preference for arthropods, annelids and fish. Predominant species included other abundant coastal and estuarine taxa, such as the shore crab *Carcinus maenas* and the amphipod *Corophium volutator*. Metabarcoding data obtained from *C. crangon*'s stomach contents also provided key insights in possible endoparasitic interactions between the shrimp and the fungus *Purpureocillium lilacinum* (phylum Ascomycota, class Ascomycetes; **chapter 4**) and can potentially be used to answer ecotoxicological questions in relation to heavy metal pollution (**chapter 5**) or could be used as a bioassessment tool to study local fish diversity (**chapter 6**).

7.2. Individual variability and survival in estuaries

Crangon crangon showed high individual variability in both its camouflage ability and prey selection during this project. This degree of flexibility might be an adaptation to the dynamic nature and large variety of habitats used by the shrimp during its life cycle (Elliott and Quintino, 2007, Wilson, 1990). During this study, shrimp were caught from a variety of estuarine soft-bottom habitats, including exposed sandy beaches, sheltered saltmarshes, sites with high shell debris, tidal pools and in ponds that are only flooded during spring tide. Animals living in these habitats are exposed to a variety of abiotic and biotic stressors which are known to influence local community structure (Sousa et al., 2007, Costa-Dias et al., 2010, Wolff, 1973). Individual flexibility in the application of anti-predator and predation strategies employed, enables shrimp to respond to the large variety of prey and predator species encountered in these habitats. As discussed in **chapter 4**, the brown shrimp is capable of obtaining its prey items by means of ambush predation, gulping of small prey and scavenging (Pinn and Ansell, 1993, Gibson et al., 1995, Tiews, 1970, Ansell et al., 1999) and will likely adapt its strategy according to food availability. To avoid predation, soft-bottom habitats provide limited shelter opportunities for demersal animals. Shelter can be sought in shallower areas, under vegetation, in turbid water or by burying, but shelter conditions vary spatially and temporally (Thrush, 1999, Ruiz et al., 1993, Pinn and Ansell, 1993, Abrahams and Kattenfeld, 1997). Instead of relying on one mechanism, *C. crangon* can avoid predation in several ways by burying, background matching and tail-flip assisted escapes (**chapter 3**; Arnott et al., 1998; 1999; Pinn and Ansell, 1993) , depending on the situation and type of predator. Overall, the variability in cryptic behaviour and trophic ecology characterised in *C.*

crangon during this project mirrors the dynamic habitat it is living in and might be one of the factors playing a role in the ecological success of this vagile estuarine crustacean (Campos and van der Veer, 2008, Gibson et al., 1993).

7.3. Hide and seek

There are clear indications that the predatory and anti-predatory behaviours of *C. crangon* are intertwined. The brown shrimp shares its habitat with several important predators and shows complex interactions with some of its prey species, e.g., flat fishes and decapods, where it acts alternately as a prey and as a predator depending on its life stage (Baeta et al., 2006, Moksnes et al., 1998, Amara, 2001, van der Veer and Bergman, 1987). Predation is a major structuring factor in the sandy beach epibenthic community (Van Tomme et al., 2014, Thrush, 1999) and to avoid predation in these soft-bottom habitats, several juvenile animals stay in the shallower upper intertidal zone where predation pressure is generally lower (Amara and Paul, 2003, Ruiz et al., 1993). The brown shrimp is one of the main predators in these upper intertidal habitats where it preys, for example, on 0-year juvenile flatfish (Amara and Paul, 2003, Beyst et al., 2001, Van Tomme et al., 2014, van der Veer and Bergman, 1987). Capture of these prey items is likely be achieved by ambush predation during which *C. crangon* remains buried just below the surface with its eyes and antennae above the sediment, ready to capture any prey within reach (Gibson et al., 1995, Pinn and Ansell, 1993). The shallow burying depth required for ambush predation increases, however, the risk of being revealed to potential prey and predators since sediment transport caused by wave and wind action can easy uncover the shrimp in these shallow upper intertidal habitats (Hewitt et al., 1997, Barshaw and Able, 1990). Camouflage, by means of background matching, can thus act as a complementary line of defence to enable the shrimp to avoid detection in dynamic habitats, while not restricting the use of their vision and olfactory senses to ambush prey (Hewitt et al., 1997, Barshaw and Able, 1990). Adult brown shrimp are also abundant in the deeper subtidal zones where sediment disturbances caused by wind and wave action might be of less concern (Kuipers and Dapper, 1984, Kuipers and Dapper, 1981). These habitats are, however, frequently visited by important predators of *C. crangon* (Amara and Paul, 2003, Beyst et al., 2001), such as sole (*Solea solea*), dab (*Limanda limanda*) and whiting (*Merlangius merlangus*), against which the combination of burying behaviour

and camouflage might provide extra protection. The ability to hide in sandy substrates to reduce depth-depended predation risks might allow *C. crangon* to utilize habitats less frequently visited by shallow-zone competitors (as shown for *Crangon septemspinosa*; Ruiz et al., 1993). The ability to camouflage itself is thus likely of major importance to *C. crangon*, both from a predator and prey perspective, and might have contributed to *C. crangon*'s ability to survive in a wide range of habitats and feed on a vast selection of prey items.

Nutrition can play an important role in the colouration and background matching ability of animals. Even though the physiological costs of background matching and other forms of animal crypsis are not fully understood (Duarte et al., 2017), studies on fish and invertebrates show that nutritional deficiencies in certain vitamins, fatty acids, lipids, carotenoids can result in the depigmentation of animals (Nicolaides and Woodall, 1962, Leclercq et al., 2009, Bolker, 2000, Styrishave et al., 2000, Kayser, 1979). During this project, even though shrimps caught in the field showed a range of colours (Figure 7.1 A,B) including red carotenoid pigment (Elofsson and Hallberg, 1973), shrimp kept in captivity on a red background failed to show an increase in red pigmentation, but dispersed their black pigment instead (Figure 7.1 C,D). While shrimp eat a very wide range of prey items in their natural environment (**chapter 4**), shrimp kept in captivity were only fed with fish tissue. Probably, this tissue was deficient in carotenoids or other compounds required for the production of red pigments (Chatzifotis et al., 2005). This nutritional dependence for camouflage could have serious implications for shrimps in the field. Starvation might, theoretically, lead to a negative feed-back loop since a lower degree of crypsis might lead to lower prey capture by ambush predation. Alternative feeding strategies such as scavenging and gulping behaviour might help reduce the risk of this negative feedback loop (**chapter 4**; Ansell et al., 1999; Tiews, 1970) . The wide range of feeding strategies applied and prey items caught by *C. crangon*, as observed during this thesis, can thus indirectly influence both its anti-predator and predatory success, indicating and complex and fascinating interaction between its hide and seek behaviour.

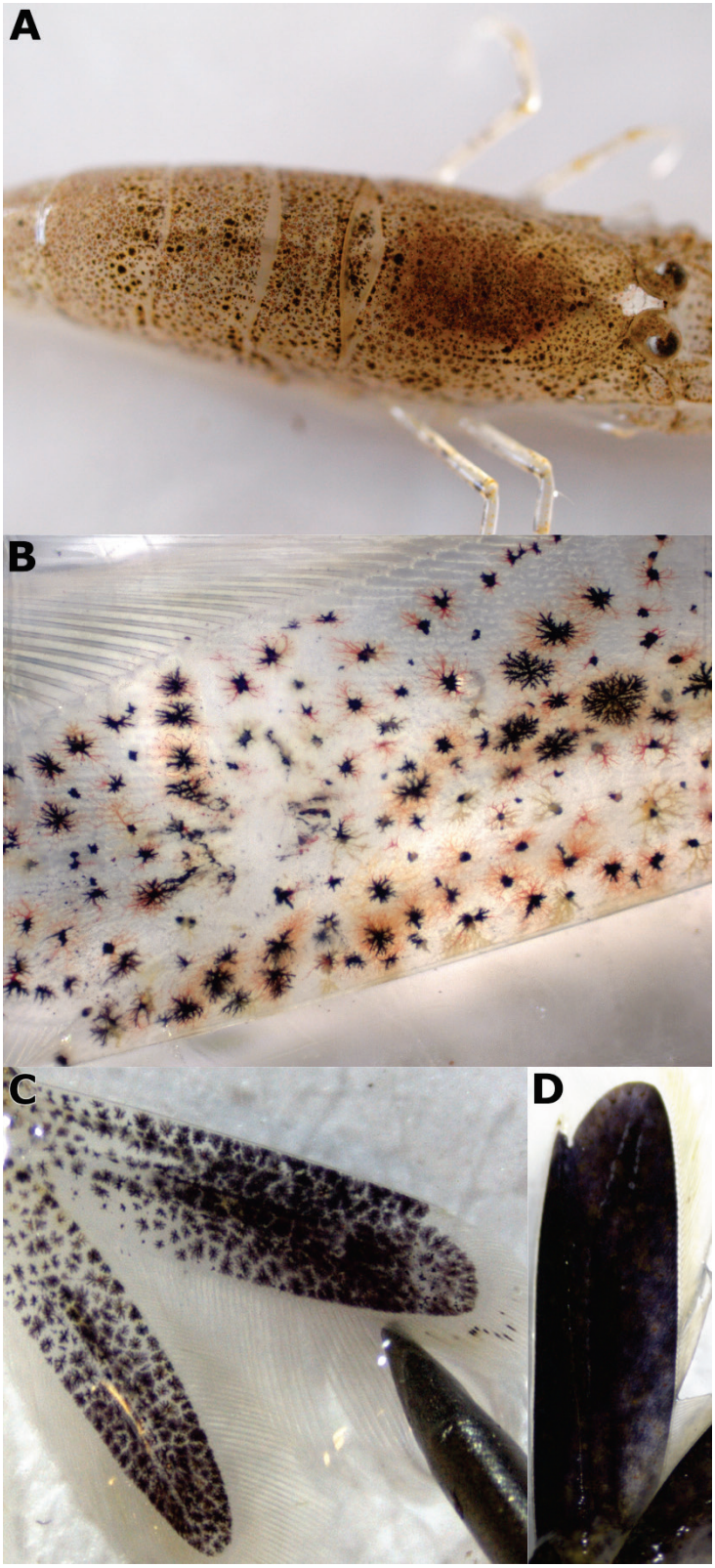

Figure 7.1. Colour variation of *Crangon crangon* under natural and laboratory conditions. A: Image of a brown shrimp taken directly after capture. B: Image of a section of the exopod of *C. crangon* (approx. 1 * 1.5 mm) kept on white sediment. This image was taken several days after capture. C: Image of an exopod of *C. crangon* (approx. 2 * 6 mm) kept on sediment consisting of a mix of colours (red, yellow, black and white). This image was taken a month after capture. Image of a section of the exopod of *C. crangon* (approx. 2 * 6 mm) kept on red sediment. This image was taken a month after capture.

7.4. Implications for ecosystem management

Successful management of marine ecosystems depends on extensive and precise knowledge on the community structure, tropic relationships, anthropogenic impacts, and ecology of species occurring in these systems. Insight in these dynamics is especially essential for fisheries target species and keystone species due to the risk of overfishing or cascading effects (Zacharias and Roff, 2001, Scheffer et al., 2005). This project focussed on the brown shrimp which is both an important target species for fisheries and a key component of European soft-bottom habitats (Campos and van der Veer, 2008, Aviat et al., 2011, Gillett, 2008, Evans, 1984). Due to its ecological and economical importance, central position in the food web, ease of collection and occurrence in anthropogenic impacted estuaries, *C. crangon* has been extensively used as a model organism in a variety of studies ranging from behavioural to ecotoxicological topics (Rodrigues and Pardal, 2014, Hellou, 2011, Smith et al., 1995, Koller, 1927). By using an integrated approach, this study provided important background information regarding fundamental knowledge of the brown shrimp, including its trophic ecology and anti-predator behaviour. Furthermore, the application of this species as an indicator species for environmental management was assessed.

The method (PIC) developed to quantify pigment cover (**chapter 2**) allowed for a simple and detailed assessment of factors playing a role in shrimp crypsis (**chapter 3**) and can be readily applied to answer essential questions in animal physiology, colouration and ecotoxicology in a wide range of species (Llandres et al., 2013, Pautsch, 1953, Reddy and Fingerman, 1995). Furthermore, the fundamental knowledge gained on the brown shrimp's anti-predator behaviour (**chapter 3**) and trophic ecology (**chapter 4**) could be used to adapt husbandry protocols (sediment, light regime, food items) for scientific and aquaculture purposes (Wennhage and Gibson, 1998, Delbare et al., 2014), in order to encourage natural (anti-predator) behaviour in a captive environment.

Data on the brown shrimp's trophic ecology (**chapter 4**), parasitology (**chapter 4**) and ecotoxicology (**chapter 5**) is essential for monitoring shrimp stock developments and health (Chávez-Sánchez et al., 2002, Ianelli et al., 2016, Van Ael et al., 2017). Even more, due to the shrimps' wide occurrence and opportunistic feeding behaviour (Campos and van der Veer, 2008, Oh et al., 2001), information gained from its stomach contents can provide essential insights in ecological, anthropogenic and ecotoxicological processes occurring in its habitat.

During this study, variation in its stomach contents was evaluated as a bioindicator tool for assessing anthropogenic impacts (**chapter 5**) and fish diversity in European estuaries (**chapter 6**). Although clear differences concerning anthropogenic impacts and fish diversity were not detected between estuaries, the results of this study clearly show the feasibility of trophic metabarcoding for the ecological assessment of marine ecosystems and the robust reconstruction of ecological networks.

The use of specific organisms to monitor ecosystem changes is a commonly used practice in ecology and environmental management. The presence, or absence, of specific key-stone or rare species has often been applied as an indicator of ecosystem health (Mills and Doak, 1993, Lawler et al., 2003). The effect of human impacts has also been monitored using specific bioindicator species, whose responses to anthropogenic pressures are considered to be representative for whole communities (Quintaneiro et al., 2006, Menezes et al., 2006). Monitoring species presence and abundance is, however, often time consuming, difficult and expensive; calling for novel techniques such as DNA metabarcoding (Bohmann et al., 2014). The power of metabarcoding to detect species based on very small amounts of DNA (Taberlet et al., 2012a) enabled the development of methods to detect prey DNA within the stomachs and faeces of predators, allowing for the use of these predators as ecological "sentinel" species for prey diversity (**chapter 6**; Bohmann et al., 2014, Schnell et al., 2015b, Schnell et al., 2012, Calvignac-Spencer et al., 2013b). Most of these studies, however, have been conducted in terrestrial habitats on carrion flies and leeches (Calvignac-Spencer et al., 2013b, Schnell et al., 2015b). This study (**chapters 5 & 6**) shows that decapod crustaceans, such as the brown shrimp, can be suitable sentinel organisms for monitoring biodiversity in marine environments, which are generally difficult to survey by traditional methods. The application of trophic metabarcoding of the stomach contents of sentinel species may therefore represent a promising tool for ecosystem management.

7.5. Future directions

By using *C. crangon* as a target organism, the present body of work provides the fundamental knowledge required to assess predator-prey interactions and verified the application of trophic molecular tools for environmental monitoring. The results of this

thesis also highlight some important open questions that remain poorly addressed and of which further research could contribute to the incorporation of predator-prey dynamics in environmental monitoring.

Firstly, the effect of predation in structuring soft-bottom communities varies considerably between studies (Van Tomme et al., 2014, Nilsson et al., 1993, Pihl and Rosenberg, 1984, Pihl, 1985, Wennhage, 2002, Feller, 2006). The study of individual plasticity might improve our understanding of these predator-prey interactions since intra- and inter-individual variation can play an important role in community dynamics (Bolnick et al., 2003, Bolnick et al., 2011). Future studies could link individual variation in crypsis and nutrition to predation success and prey choice to enhance understanding of the intra- and inter-individual variation observed in predator-prey interactions in sandy habitats. This could, for example, be achieved by combining behavioural methods such as PiC with molecular tools investigating the diet and expression of genes related to the motile activities of pigments in chromatophores (San-Jose and Roulin, 2017, Fujii, 2000).

Secondly, HTS techniques still suffer from limitations caused by variations caused by biological factors, sequencing errors and bioinformatic challenges (Alberdi et al., 2018, Taberlet et al., 2012a, Coissac et al., 2012). Especially the high uncertainty in the use of relative abundances and the inability of detecting cannibalism by trophic barcoding were two major limitations encountered during this thesis. Although much progress has already been achieved to address some of these issues (e.g. Deagle et al., 2006, Deagle and Tollit, 2007, Thomas et al., 2016, Alberdi et al., 2018), more methodological studies are required to improve the reliability of trophic metabarcoding before it can be successfully applied for (estuarine) biomonitoring studies.

Thirdly, anthropogenic impact assessments in dynamic systems such as estuaries are challenging due to the large natural variations in abiotic and biotic components of these systems. Even more, confounding effects of these natural variables on the bioavailability and accumulation of pollutants are known to complicate any conclusions drawn on correlations between community variation and individual pollutants (Holmstrup et al., 2010, Bryan and Langston, 1992). Organisms living in estuaries are naturally adapted to high levels of environmental stress, making the detection of anthropogenic stress even more difficult

(Elliott and Quintino, 2007). Thus, while the results of this study clearly show the potential of applying shrimp stomach contents as a bioindicator for environmental change, more research is required to enhance the reliability and further development of this tool. As discussed in **chapter 6**, this study provides the tools for future studies to develop molecular trophic ecotoxicological assessments to be conducted in controlled environments such as mesocosm systems or enclosed pools. Results of these studies should, furthermore, be confirmed by laboratory tests using individual and combined stressors since many anthropogenic stressors can have synergistic effects (Crain et al., 2008).

7.6. Final conclusions

Survival in dynamic systems such as estuaries calls for a vast level of flexibility in dealing with anthropogenic and natural stressors. Estuarine animals are highly adapted to handle variation in both the abiotic and biotic components of these systems. Adaptations to obtain crypsis or acquire sufficient nourishment are deeply interlinked in these habitats and can provide fundamental information on the evolution of highly flexible strategies, such as background matching or trophic opportunism. By studying the anti-predator behaviour and trophic ecology of *C. crangon*, this thesis provides key insights in identifying the major environmental and behavioural factors influencing the evolution of animal background matching and contributes towards a more robust reconstruction of ecological estuarine networks. The insights gained on its behaviour, trophic relationships and ecotoxicology are of consequence for the perception and management of the whole estuarine system, given the pivotal role of *C. crangon* in European coastal waters.

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Appendix 1. Glossary of terms

Abundance renormalization: The process of calculating relative abundances from read numbers
Akaike information criterion (AIC): Criterion that represents the relative quality of a statistical model for a given set of data. Relative AIC is applied for model selection
Aluminium-normalisation: Representation of heavy metal concentrations as metal:Aluminium ratios to correct for natural differences in mineralogy and granulometry between sites
Amplicon sequencing: Targeted sequencing of an amplified marker gene.
Anthropogenic stressors: Human induced stressors
Background matching: Crypsis obtained by generally matching the colour, lightness or pattern of the background
Beta distribution: Probability density function for data that is bounded between 0 and 1
Bioaccumulation: The accumulation of substances in an organism
Biomarker: A biological response measured by endpoints from molecular to behavioural levels, providing exposure and/or effect evidence of increasing concentrations of a specific pollutant or a group of pollutants
Biorhythm: A daily cycle of a biological process, based on 24-hour intervals. A biorhythm does not have to be under endocrine control
Blocking primer: Predator-specific blocking oligonucleotides that reduce amplification of the predator DNA
Carapace length: Distance between the posterior margin of the eye socket and the posterior dorsal margin of the carapace
Chimeras: A single cDNA sequence originating from two different DNA transcripts
Chromatophore index: Index based on the melanophore index used for chromatophores that contain other pigments than melanin
Chromatophores: Cells containing pigmented organelles
Chromatosomes: Clusters of two or more chromatophores which can be of different colours or of the same colour. These chromatophores can overlap partially or may be superimposed
Circadian rhythm: A daily cycle of a biological process, based on 24-hour intervals, that is under endocrine control
Community DNA: DNA derived from many individuals representing multiple species
Conspecifics: Organisms of the same species
Crypsis: Preventing detection by other organisms
Degenerate primers: A mixture of amplicon sequencing with high similarity, but in which some positions contain a number of possible bases, to cover all possible nucleotide combinations for the targeted gene(s)
Demultiplexing: Dividing sequence reads into separate files for each index tag
Ecological dominance: The degree that a certain taxon is more numerous than other taxa in a community.
Effect of metal: Deleterious consequences of metal exposure to organisms
Environmental DNA: All DNA molecules (community DNA and extra-organismal DNA) present in an environmental sample, collected either in the organisms or in their habitat.
Epibenthic Organisms: Organisms living on the surface of the bottom of a water body
Essential metals: Metals that are a necessary part of the nutrition and physiology of organisms
Euryhaline species: Species that can withstand a wide range of salinities (In contrast to stenohaline species).
Exopod: Outer uropod (last pair of abdominal appendages of the tail fan *Crangon crangon* and other crustaceans)
Facultative protandric hermaphrodite: Sequential hermaphroditism where organisms are born male but are capable in changing sex to female and at some point in their lifespan
Generalist predator: A species that feeds on a wide range of prey items throughout their life cycle
Heavy metal: A potentially toxic metal with a relatively high density (more than 5 g/cm^3) or high atomic weight.
High-throughput sequencing: A process where many millions of sequences are generated simultaneously. Also called next generation sequencing.
Image segmentation: Partitioning an image into sets of pixels
Inosine nucleotides: Nucleotides that can match any nucleotide
Intermoult: Period between consecutive moults during which no growth takes place
Macrofauna: Invertebrates that live on or in sediment, or attached to hard substrates, with body size of >1 mm, although this size fraction often varies across studies.

Meiofauna: A loose term to define metazoan species with a body size <1 mm, although this size fraction often varies across studies.

Melanophore index: Index designed by Hogben and Slome (1931) that classifies melanophores based on their pigment dispersion

Melanophore: Chromatophore containing the pigment melanin

Metabarcoding: The identification of multiple taxa based on DNA extracted from a collection of organisms or environmental samples by means of next generation sequencing using taxonomically general gene-specific PCR primers

Molecular marker: A DNA sequence targeted in amplicon sequencing

Morphological colour change: Slow colour changes (days to months) by means of changes in the amount of pigments, or by modification of these pigments or other colour components

Moulting: Shedding of the exoskeleton to allow for new growth. Also called Ecdysis

Nursery habitat: Habitats with a greater density, survival rate and growth of juveniles compared to surrounding habitats

Opportunist predator: A species who's diet reflects the local prey community

Overdispersion: The presence of greater variability in a data set than would be expected based on a given statistical model

Physiological colour change: Fast colour changes (milliseconds to hours) achieved by physiologically by changing the distribution of pigments, microstructures or the refractive index of layers in the integument

Pigment cover: Surface of a defined area of an animal body covered by specific pigments

Polymerase chain reaction: Method to generate multiple copies of a particluar DNA sequence by means of repeated reactions with a polymerase

Primary response: The chromatophore reacts directly to the intensity of incident light. This response is not mediated by the eye or controlled by neuroendocrine factors

Primer: A short strand of DNA or RNA that is hybridized to a specific section of target DNA to form the starting point of DNA replication in a polymerase chain reaction

Reference estuary: Estuary with a relatively low level of anthropogenic disturbance

Secondary predation: prey material present in the stomach of consumed prey

Secondary response: Degree of pigment dispersion determined by the ratio of light directly incident on the eye, to the quantity of light received by the eye after reflection from the background. This response to light is mediated by the eye and controlled by neuroendocrine factors

Stragglers: Taxa found in estuaries which normally occur in marine or freshwater habitats

Tag-switching: Sequences in metabarcoding sequencing outputs with false combinations of used tags

Thresholding: Method to perform image segmentation in a graphic editing program

Total length: Distance between the tip of rostrum to the tip of telson

Total metal concentration: Concentration of metal measurable in water, sediment or tissue after acid digestion. Total metal concentration can include dissolved metals, metal precipitates, metals associated with the mineral lattice and metals absorbed to/into sediment, organic matter and tissue, and can consist of a mix of chemical forms of the same element.

Trophic interactions: Interactions between different levels within a food web

Trophic metabarcoding: Metabarcoding of DNA extracted from animal gut/stomach, regurgitated or faeces samples in order to study trophic relationships between species

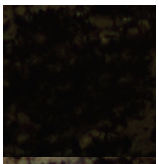
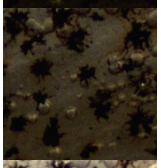
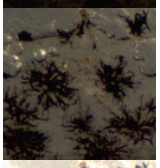
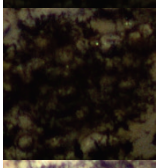
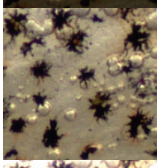
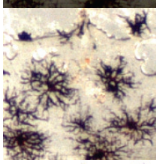
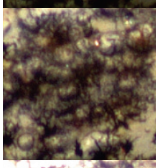
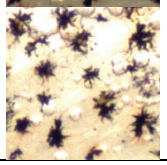
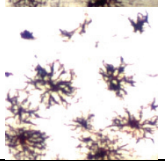
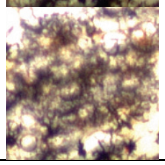
Uropod: Appendixes of the tail section of *Crangon crangon* (and other crustaceans)

Wobble bases: Equimolar mixtures of two or more different bases at a given position

Appendix 2. Supplementary material for chapter 2

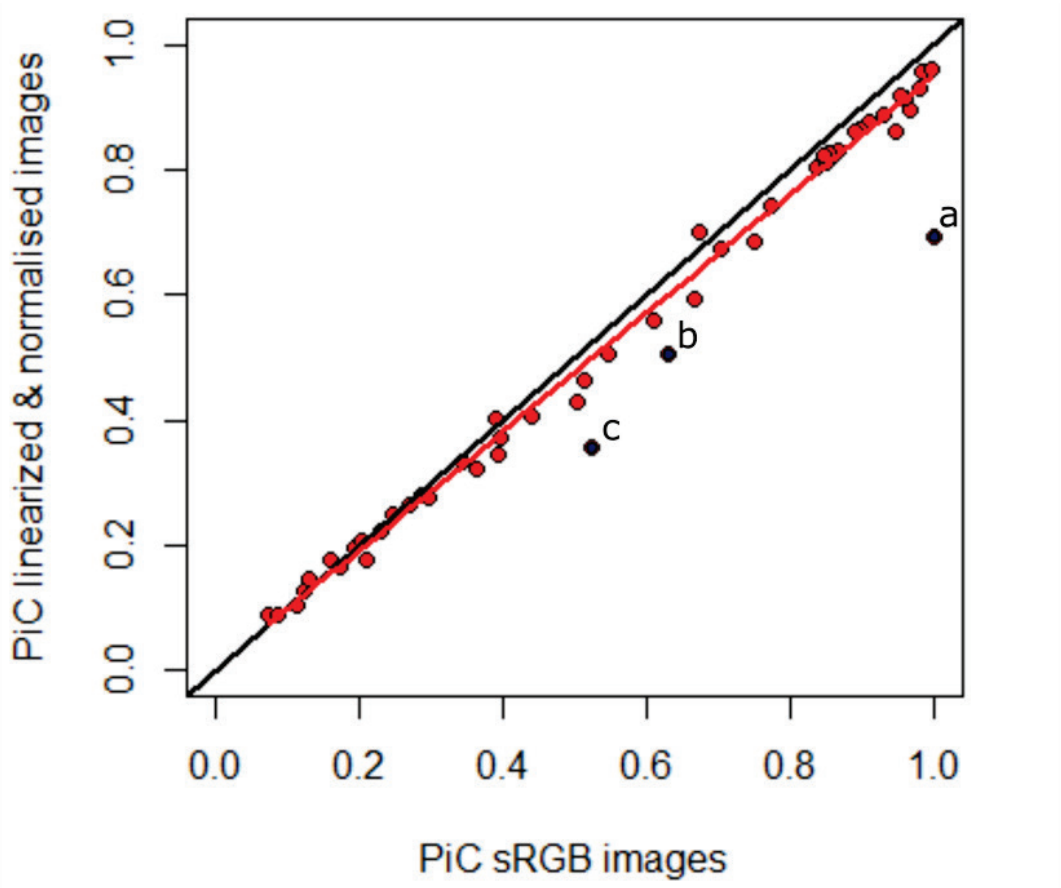
SUPPORTING TABLES

Appendix 2.1. Effect of illumination on measured dark pigment cover (PiC**).** *Crangon crangon* (N = 3) were photographed on a white background, illuminated by two led spotlights (JANSJÖ; 88 lm; 3000 Kelvin) for different exposure times. PiC was analysed using the IsoData thresholding algorithm without manual adaptation.

	Shrimp 1		Shrimp 2		Shrimp 3	
Exposure (ms)	Image	PiC (%)	Image	PiC (%)	Image	PiC (%)
10		24		33		63
20		22		33		66
50		22		30		68
100		19		15		46

Appendix 2.2 Regression parameters describing the relationship between chromatophore index and dark pigment cover fraction for three observers.

Observer	R^2	Intercept	Slope
1	0.934	-3.1625	0.61736
2	0.9125	-3.3798	0.65672
3	0.9444	-3.2235	0.63221

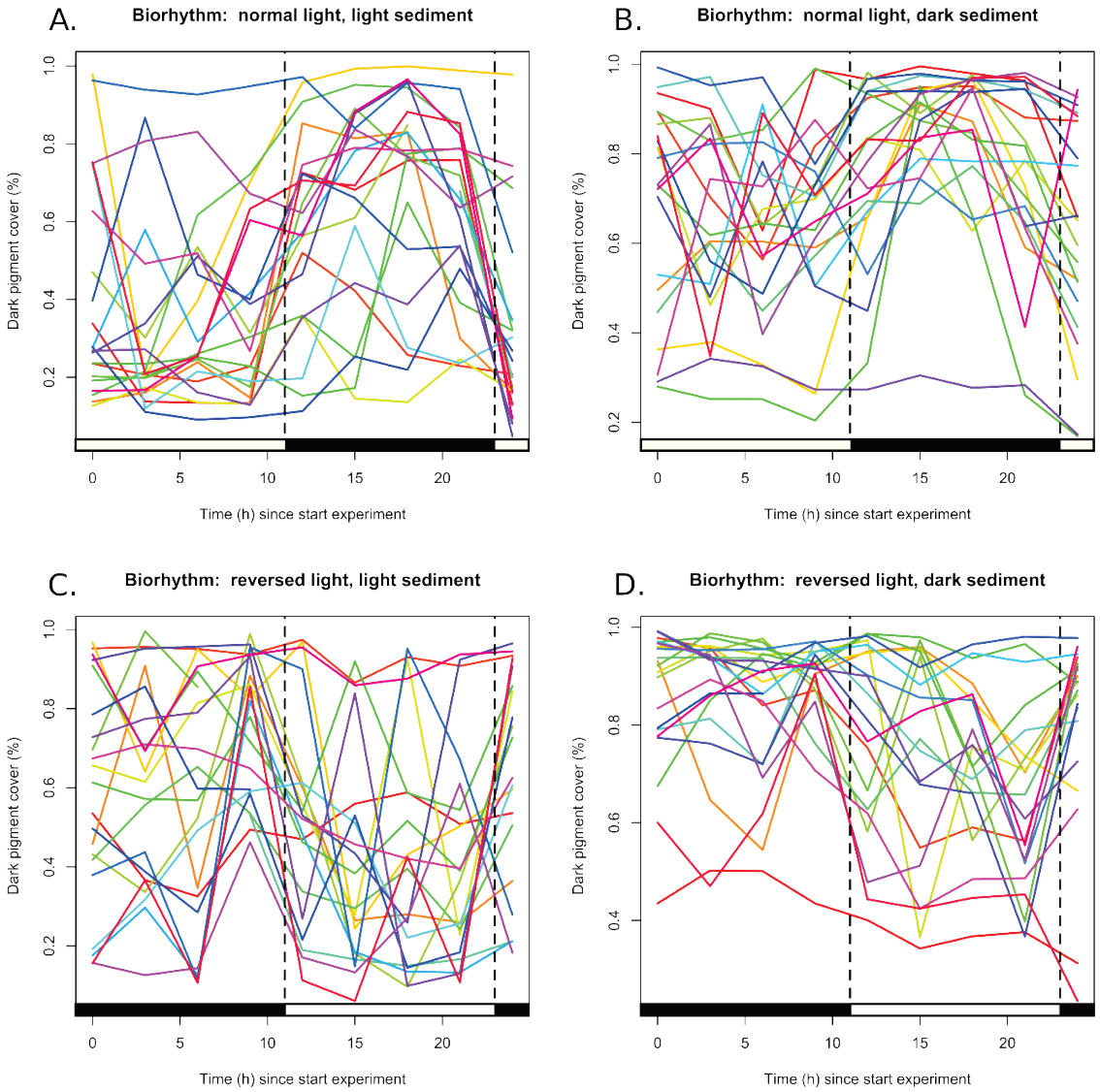


Appendix 2.3. Relationship between the dark pigment cover fraction (PiC) with and without linearization and normalisation of 50 sRGB colour images of *Crangon crangon*'s exopods. The red line shows the linear regression fit. Three outliers (a-c) were identified and removed prior to analysis, since the thresholding algorithm could not produce reliable PiC estimates without manual adaptation (due to image quality and exposure, as confirmed by visual observation).

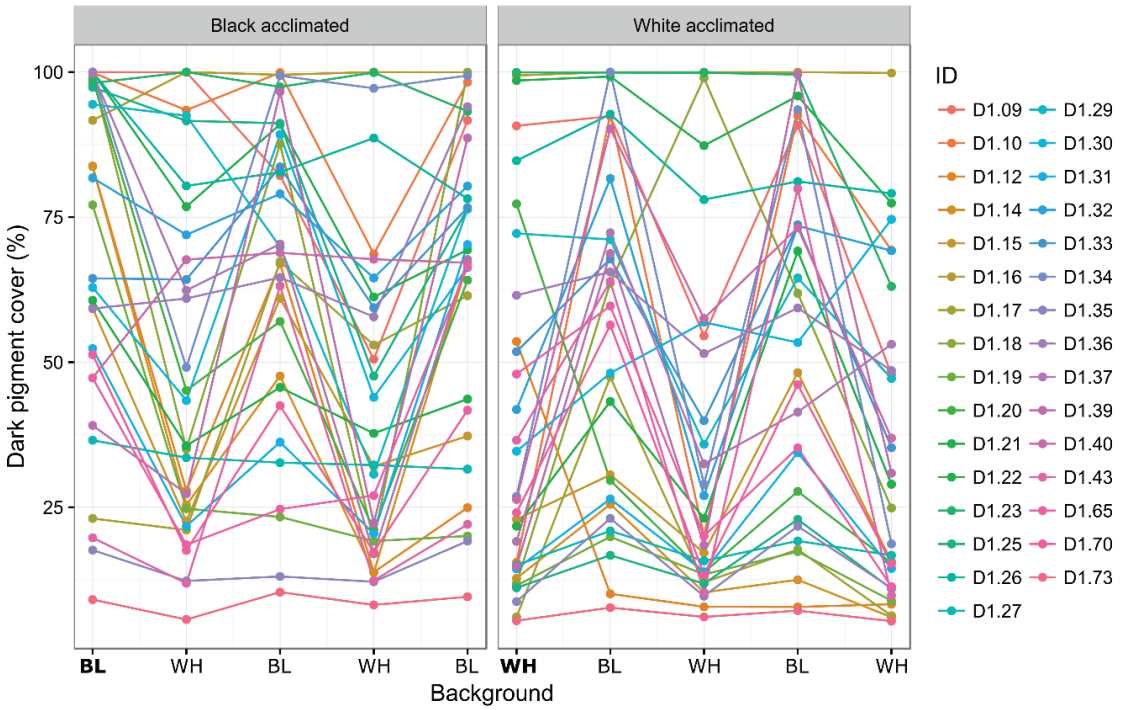
Appendix 3. Supplementary material for chapter 4

Appendix 3.1. Biorhythm model coefficient estimates. Coefficients estimates are shown for the model incorporating sediment colour (white vs. black), artificial illumination (on vs. off), presence of day light, time since change of the light regime (TLC) and the interaction between artificial Illumination and TLC.

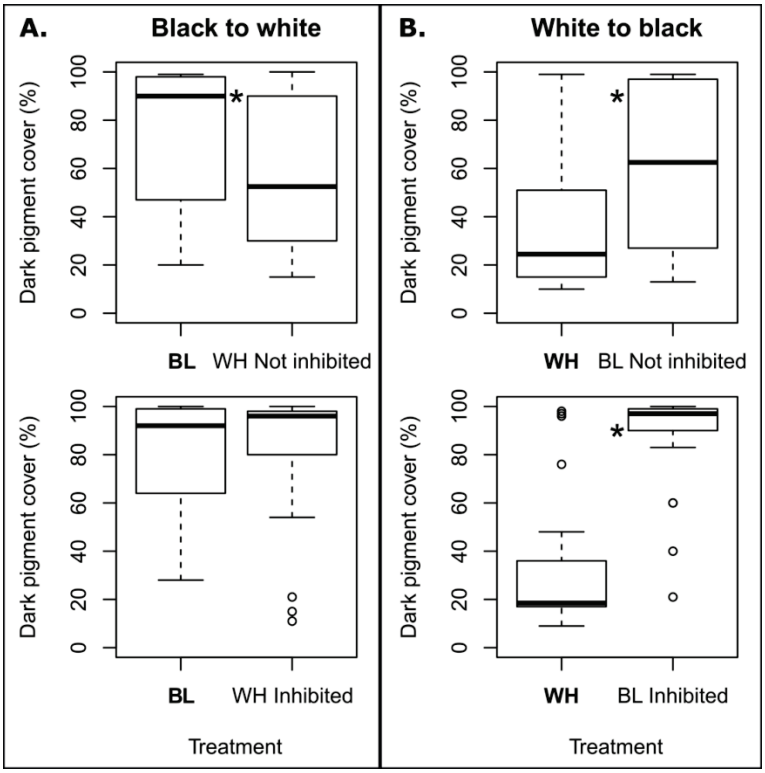
Factor	Coefficient (±SE)	z-value	P
Intercept	1.58 ± 0.15	10.70	< 0.0001
Illumination (on)	-0.56 ± 0.10	-5.38	< 0.0001
Sediment colour (white)	-1.06 ± 0.17	-6.07	< 0.0001
TLC	0.04 ± 0.01	3.15	0.0016
Daylight	-0.25 ± 0.06	-3.86	0.0001
Illumination (on) : TLC	-0.05 ± 0.02	-3.18	0.0015



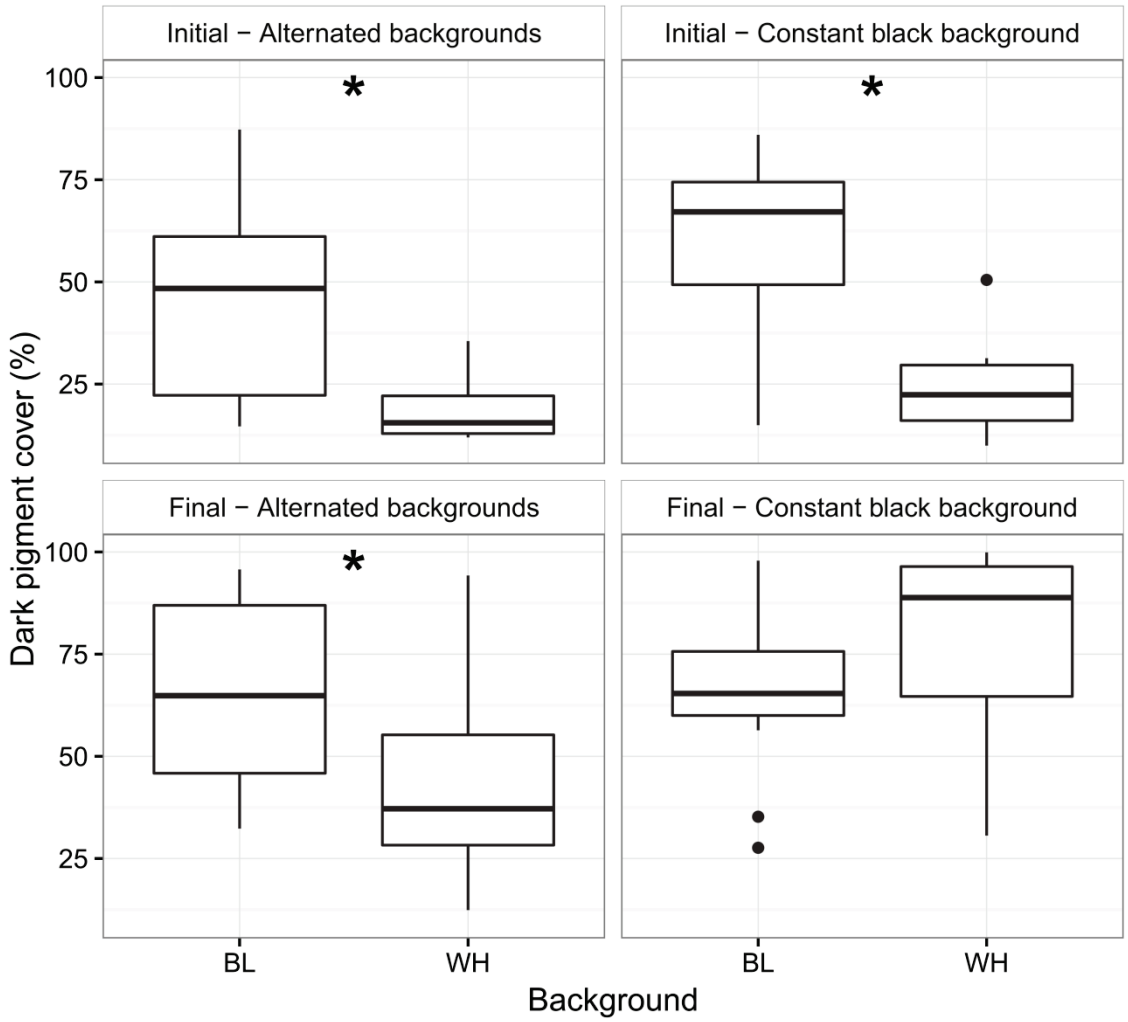
Appendix 3.2. Effect of background colour and light on individual *C. crangon* dark pigment cover over a day-night cycle. The illumination regime is indicated with a black/white bar below each graph. Each line represents a different individual. Dashed vertical lines: light switch. Time = 0 is 9:00am.



Appendix 3.3. Dark pigment cover (%) of individual *C. crangon* (N = 33) during repeated shifts between black (BL) and white (WL) backgrounds. First measurement (in bold) was performed after 24h acclimation and all subsequent measurements after 1 hour permanence on the respective background. Each colour represents a different individual (ID).



Appendix 3.4. Box-and-whisker plots showing dark pigment cover (%) of *C. crangon* that were inhibited or not inhibited from burying on black (BL) or white (WH) sediment. A: Shrimp were acclimated on black sediment (bold) and moved to white one hour (N = 25). B: Shrimp were acclimated on white sediment (bold) and moved to black for one hour (N = 22). *: $P < 0.05$ (Wilcoxon Signed Rank Test).



Appendix 3.5. Box-and-whisker plots showing dark pigment cover (%) of *C. crangon* kept on constant black background (N = 11) or on alternating black and white backgrounds (N = 15) for 21 days. At day 0 (Initial) and day 21 (Final), shrimp were acclimated on a black (BL) background for 24h and moved to a white (WH) for 1 hour. *: $P < 0.05$ (Wilcoxon Signed Rank Test).

Appendix 4. Supplementary material for chapter 4

Appendix 4.1. Number of pooled full stomachs per sample. Only samples with less than 8 pooled stomachs are shown.

Estuary	Sample	N stomachs
Aveiro	Av_1C	4
Aveiro	Av_2B	7
Eastern Scheldt	ES_2A	5
Eastern Scheldt	ES_2B	4
Eastern Scheldt	ES_3C	5
Kent	Ke_1C	5
Kent	Ke_4C	7
Mersey	Me_1C	4
Mersey	Me_2A	6
Mersey	Me_3B	7
Western Scheldt	WS_2C	4
Western Scheldt	WS_4C	4

Appendix 4.2. Mean (±SD) total length of *C. crangon* (>20 mm TL) sampled and dissected for stomach DNA extraction per site.

		Sampled		DNA extracted	
Estuary	**Site**	**Mean (mm)**	**SD**	**Mean (mm)**	**SD**
Aveiro	Av_1	32	5	32	6
Aveiro	Av_2	30	4	30	3
Aveiro	Av_3	31	4	31	5
Eastern Scheldt	ES_1	25	6	27	6
Eastern Scheldt	ES_2	33	7	32	6
Eastern Scheldt	ES_3	30	6	32	6
Eastern Scheldt	ES_4	34	7	34	8
Kent	Ke_1	32	3	31	3
Kent	Ke_2	32	3	32	4
Kent	Ke_3	31	3	32	3
Kent	Ke_4	29	2	29	2
Mersey	Me_1	47	4	48	3
Mersey	Me_2	40	7	42	7
Mersey	Me_3	40	7	41	7
Mersey	Me_4	39	7	36	5
Mersey	Me_5	41	7	40	6
Mersey	Me_6	42	7	40	4
Minho	Mi_1	41	4	39	3
Minho	Mi_2	39	5	38	4
Minho	Mi_3	35	5	36	3
Western Scheldt	WS_1	32	5	32	4
Western Scheldt	WS_2	26	4	27	4
Western Scheldt	WS_3	36	6	37	6
Western Scheldt	WS_4	44	6	43	7

Appendix 4.3. *Purpureocillium lilacinum* sequences detected in *C. crangon* stomach samples

Marker	Sequence	Match	Query cover	E-Value	Identity
COI	TTTATCAGGATTACAAAGTCACAGTG GACCTAGTGTAGATTTAGCAATTTTT GCTTTACACCTTTCAGGGGTAAGTAG TTTATTAGGAGCAATAAACTTCATAA CTACAATCGCTAATATGAGAACACCA GGAATAAGATTACACAAATTAGCCTT ATTCGGGTGAGCTGTAGTTATAACA GCTATCTTATTATTATTATCATTACCT GTTTTAGCTGGAGGTATTACAATGGT ATTAACAGATAGAAATTTTAACACTT CATTCTTCGAAGTAGCTGGTGGTGG AGATCCTATATTATTCCAACACTTATT C	*Purpureocillium lilacinum*	100%	$2e^{-156}$	99%
ITS	ACTCCCAAACCCACTGTGAACCTTAC CTCAGTTGCCTCGGCGGGAACGCCC CGGCCGCCGGCCCCCGCGCCGGCGC CGGACCCAGGCGCCCGCCGCAGGG ACCCCAAACTCTCTTGCATTACGCCC AGCGGGCGGAATTTCTTCTCTGAGTT GCACAAGCAAAAACAAATGAATCAA AACTTTCAACAACGGATCTCTTGGTT CTGGCATCGATGAAGAACGCAGCGA AATGCGATAAGTAATGTGAATTGCA GAATTCAGTGAATCATCGAATCTTTG AACGCACATTGCGCCCGCCAGCATTC TGGCGGGCATGCCTGTTCGAGCGTC ATTTCAACCCTCGAGCCCCCCCGGGG GCCTCGGTGTTGGGGGACGGCACAC CAGCCGCCCCCGAAATGCAGTGGCG ACCCCGCCGCAGCCTCCCCTGCGTA	*Purpureocillium lilacinum* isolate NIOSN_SK56_S76	100%	0.0	100%

Appendix 4.4. Pairwise comparisons (Bonferroni-corrected) in number of reads (log-transformed) per sample between estuaries. One-way ANOVA: Estuary: $F_{5,42}$: F = 3.551, P < 0.01; Sites nested in estuary: $F_{18,42}$: F = 1.064, P < 0.41.

P-values of Bonferroni-corrected pairwise t-tests

	Av	ES	Ke	Me	Mi
S	1	-	-	-	-
Ke	1	1	-	-	-
Me	1	1	1	-	-
Mi	0.6735	0.0024*	0.054	0.1473	-
WS	1	1	1	1	0.12

* P < 0.05; Av = Aveiro, ES = Eastern Scheld, Ke = Kent, Me = Mersey, Mi = Minho, WS= Western Scheld

Appendix 4.5. Differences in relative mean read abundance of detected phyla in sediment and *C. crangon* full stomach samples. Samples are averaged per site (N=24) and tested with a paired Wilcoxon signed-rank test. **: P < 0.01

	Mean relative abundance (%)		Wilcoxon signed-rank test		
Phylum	**Sediment**	**Full Stomach**	**W**	**P**	
Annelida	3.30	13.28	241	0.008	**
Arthropoda	5.12	45.83	299	0.000	**
Bacillariophyta	19.74	2.26	5	0.000	**
Chlorophyta	0.22	1.03	265	0.000	**
Chordata	0.08	4.41	208	0.000	**
Cnidaria	0.79	1.92	163	0.726	
Dinoflagellata	5.88	1.48	54	0.005	**
Discosea	10.67	0.20	0	0.000	**
Mollusca	0.16	1.22	214	0.001	**
Oomycota	2.95	0.29	3	0.000	**
Rhodophyta	2.11	1.65	107	0.229	
Unassigned	44.62	22.36	11	0.000	**

Appendix 4.6. Pairwise comparisons (Bonferroni-corrected) of square-root transformed Bray-Curtis dissimilarities of *C. crangon* pooled stomach contents between estuaries. * = P < 0.05; ** = P < 0.01.

Pairs	F.Model	R^2	P	
Eastern Scheldt vs Western Scheldt	1.576	0.073	1.000	
Eastern Scheldt vs Minho	3.744	0.172	0.015	*
Eastern Scheldt vs Aveiro	2.693	0.137	0.387	
Eastern Scheldt vs Mersey	2.642	0.096	0.070	
Eastern Scheldt vs Kent	5.327	0.210	0.006	**
Western Scheldt vs Minho	2.961	0.141	0.058	
Western Scheldt vs Aveiro	2.112	0.111	0.259	
Western Scheldt vs Mersey	1.449	0.055	1.000	
Western Scheldt vs Kent	4.180	0.173	0.028	*
Minho vs Aveiro	5.429	0.266	0.007	**
Minho vs Mersey	2.780	0.108	0.024	*
Minho vs Kent	5.749	0.242	0.003	**
Aveiro vs Mersey	3.522	0.138	0.030	*
Aveiro vs Kent	6.312	0.271	0.034	*
Mersey vs Kent	2.478	0.090	0.286	

Appendix 4.7.. Mean (± SD) Salinity, median grainsize and total organic matter (TOM) per estuary

Estuary	Salinity	Grain Size	TOM
Aveiro	25.5 (± 8.5)	271 (± 151)	1.32 (± 0.96)
Eastern Scheldt	31 (± 0.2)	294 (± 85)	0.28 (± 0.11)
Kent	0.3 (± 0.1)	76 (± 6)	0.92 (± 0.35)
Mersey	17.6 (± 10.2)	132 (± 90)	2.6 (± 3.47)
Minho	6.5 (± 3.5)	477 (± 181)	0.99 (± 1.15)
Western Scheldt	23.2 (± 5.6)	259 (± 101)	0.81 (± 0.57)

Appendix 5. Supplementary material for chapter 5

Appendix 5.1. Percentage (%) recovery of elements in certified reference materials (CRM) after nitric acid digestion and analysis by ICP-OES.

CRM	As	Cd	Cr	Cu	Ni	Pb	Zn	N samples
ERM-CE28k	198	2030*	UD	166	UD	1294	68	4
PACS-2	108	UD	67	109	78	143	117	6

UD: Reference is value under the detection limit; *: Reference value is under the lowest standard used for ICP-OES analysis.

Appendix 5.2. Number of reads used for MOTU richness rarefaction per DNA sample substrate and taxonomic group.

DNA sample substrate	Taxonomic group	Minimum number of reads (prior to sample removal) per site	Number of samples removed	Number of reads sampled per site
Sediment	All MOTU	5706	0	4281
	Annelida	11 (3)	1	8
	Arthropoda	66	0	50
Stomach	All MOTU	5088	0	3800
	Annelida	49	0	37
	Arthropoda	92	0	70

Appendix 5.3. Mean (±SD) values of environmental values per estuary.

Estuary	Temperature	Salinity	pH	Grain Size	Nitrate	TOM
Kent	15.88 ± 0.87	0.33 ± 0.08	7.80 ± 0.23	76.36 ± 6.42	3.34 ± 1.07	0.92 ± 0.35
Mersey	17.45 ± 0.78	17.41 ± 10.60	8.24 ± 0.08	150.59 ± 81.08	11.54 ± 8.51	1.54 ± 2.37
Aveiro	20.45 ± 0.30	25.53 ± 8.47	8.51 ± 0.11	270.82 ± 151.44	0.64 ± 1.11	1.32 ± 0.96
Minho	17.90 ± 3.98	6.45 ± 3.54	7.59 ± 0.11	477.24 ± 181.19	8.15 ± 5.33	0.99 ± 1.15
Eastern Scheldt	17.85 ± 0.56	30.91 ± 0.19	8.67 ± 0.19	259.28 ± 61.48	0.00 ± 0.00	0.28 ± 0.14
Western Scheldt	18.30 ± 0.88	23.2 ± 5.64	8.62 ± 0.40	259.03 ± 101.23	1.01 ± 0.96	0.81 ± 0.57

Appendix 6. Supplementary material for chapter 6

Appendix 6.1. Number of *C. crangon* stomachs pooled per sample.

	Estuary	Sample	Site	N stomachs
Netherlands	Eastern Scheldt	1	ES1	24
		2	ES2	21
		3	ES3	24
	Western Scheldt	1	WS1	24
		2	WS2	20
		3	WS3	17
		4	WS4	20
Portugal	Minho	1	Mi1	24
		2	Mi2	24
		3	Mi3	24
	Aveiro	1	Av1	20
		2	Av2	23
		3	Av3	16
United Kingdom	Mersey	1	Me1	16
		2	Me2	24
		3	Me3	24
		4	Me4	24
	Kent	1	Ke1	21
		2	Ke2	16
		3	Ke3	24
		4	Ke4	23
	Tees*	1	Te1	5
		2	Te1	5
	Tweed*	1	Tw1	8
		2	Tw1	8
		3	Tw1	4

* Multiple biological replicates were taken from one site

Appendix 6.2. Summary of the bioinformatic pipelines used for both markers. owi_recount_swarm and owi_add_taxonomy are custom R scripts available at http://github.com/metabarpark.

	PIPELINE FOR COI	**PIPELINE FOR 12S**
PCR amplification	Leray-XT primers. Single PCR protocol. Three amplifications per site (6- 8 stomachs)	MiFish primers. 2-PCR protocol. 2 replicates per sample. One amplification per site (16-24 stomachs)
Library preparation	NEXTflex (BIOO). Separate libraries for sediments and stomach samples.	NEXTflex (BIOO). 2 replicate libraries.
HT Sequencing	Illumina MiSeq 2x250 bp	Illumina MiSeq 2x150 bp
Raw sequences QC	fasttqc fastx_trimmer	fasttqc No trimming needed
PE alignment	illuminapairedend	illuminapairedend
Demultiplexing	obiannotate/obisplit ngsfilter	obiannotate/obisplit ngsfilter
Length filter	obigrep 300-320 bp	obigrep 140-190 bp
Dereplication	obiuniq	obiuniq
Rename identifiers	obiannotate	obiannotate
Chimera removal	vsearch uchime_denovo	vsearch uchime_denovo
Clustering	SWARM v2 d=13 obitab owi_recount_swarm delete singletons	SWARM v2 d=3 obitab owi_recount_swarm delete singletons
Taxonomic assignment	ecotag using db COI Sep2017	ecotag using db Miya Sep2017
Add higher taxa	owi_add_taxonomy	owi_add_taxonomy
Final refinements	Blank correction Removal of non-fish MOTUs Abundance renormalization Collapse multi-MOTU species Minimal abundance filtering (>4 reads) Collapse biological replicates per site by adding abundances	Blank correction Removal of non-fish MOTUs Abundance renormalization Removal of MOTUs detected in just one of the replicates (>1 read per sample) Collapse replicates by adding abundances

Appendix 6.3. Results of the generalized linear model testing the effects of the number of pooled stomachs per sample, country and estuary nested within country on the number of MOTUs detected in the DNA of *C. crangon* pooled stomach samples amplified with the MiFish (12S) primer pair. * = P <0.05

Predictor	Coefficient estimate	SE	Z	P
Intercept	0.79	0.78	1.03	0.305
Number of stomachs	0.02	0.04	0.57	0.567
NL	0.72	0.40	1.81	0.070
UK	0.54	0.39	1.40	0.163
PT: Minho	1.01	0.39	2.56	**0.010***
NL: Eastern Scheldt	0.14	0.29	0.49	0.626
UK: Kent	-0.11	0.30	-0.37	0.709

NL: Netherlands; PT: Portugal; UK: United Kingdom. *: P < 0.05

Appendix 6.4. Results of the generalized linear model testing the effects of country and estuary nested within country on the number of MOTUs detected in the DNA of sediment samples amplified with the MiFish (12S) primer pair. * = P <0.05

Predictor	Coefficient estimate	SE	Z	P
Intercept	1.54	0.27	5.76	**0.000***
NL	0.36	0.35	1.02	0.306
UK	0.33	0.33	1.00	0.318
PT: Minho	0.13	0.37	0.37	0.715
NL: Eastern Scheldt	0.24	0.28	0.86	0.389
UK: Kent	0.35	0.26	1.38	0.168

NL: Netherlands; PT: Portugal; UK: United Kingdom. *: P < 0.05

Appendix 6.5. Results of the generalized linear model testing the effects of the number of pooled stomach per sample, country and estuary nested within country on the number of MOTUs detected in the DNA of *C. crangon* pooled stomach samples amplified with the Leray-XT (12S) primer pair.

Predictor	Coefficient estimate	SE	Z	P
Intercept	0.13	1.42	0.10	0.924
Number of stomachs	0.03	0.07	0.41	0.679
NL	-0.78	0.74	-1.06	0.288
UK	-0.35	0.60	-0.59	0.555
PT: Minho	1.03	0.55	1.90	0.058
NL: Eastern Scheldt	0.64	0.71	0.89	0.373
UK: Kent	0.03	0.58	0.05	0.960

NL: Netherlands; PT: Portugal; UK: United Kingdom.